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(54) Title: THERMOSTABLE MUTL GENES AND PROTEINS AND USES THEREFOR (57) Abstract <p>Isolated nucleic acids which encode a thermostable protein that enhances specific binding of a thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid and recombinant vectors comprising nucleic acid which encodes a thermostable protein that enhances specific binding of a thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid are disclosed. Also disclosed are isolated thermostable proteins that enhance specific binding of a thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid and host cells comprising a recombinant gene which can express a thermostable protein that enhances specific binding of a thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid. Further disclosed are methods of reducing DNA misincorporation in an amplification reaction, methods for detecting a nucleic acid which includes a specific sequence, methods for amplifying a nucleic acid comprising a specific sequence, and methods for selecting against a nucleic acid comprising a specific sequence.</p>		

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THERMOSTABLE MUTL GENES
AND PROTEINS AND USES THEREFOR

Description

Background of the Invention

5 The polymerase chain reaction (PCR) is one of the most important technologies for genome analysis. One of the weaknesses of PCR is that primer extension from mismatched primers occurs. Extension from mismatched primers limits allele-specific amplification and detection of mutations and polymorphisms to some extent with homogeneous DNA samples (e.g. for genotyping), but to a greater extent for heterogeneous DNA samples (e.g. for detection of cancer mutations). Another of the weaknesses of PCR is much poorer fidelity than observed during *in vivo* DNA replication, as reflected in (1) a rather high rate of nucleotide misincorporation, leading to difficulty in using PCR for faithful cloning and (2) the production of multiple bands when di- and trinucleotide repeats are amplified. An order of magnitude improvement in PCR specificity and fidelity could increase accuracy in genotyping and somatic mutation detection and open up new uses for PCR, including the reproducible and faithful cloning of genomic DNA fragments up to several kilobases in length. The present invention provides such an improvement in PCR.

25 The ligase chain reaction (LCR) and its variations (e.g., oligonucleotide ligation assay (OLA), ligase detection reaction (LDR)) are alternative techniques for genome analysis. A commonly recognized source of spurious background signal in LCR and its variations, as well as in PCR and its variations, is the hybridization of an oligonucleotide such as a probe or a primer, to regions of

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the nucleic acid not intended to be amplified. Generally, these hybridizations occur because the target sample contains, in addition to the target sequence itself, other sequences with some similarity to the target nucleic acid.

5 Although hybridization of probe or primer to these similar sequences is not as probable as to the target sequence, some hybridization can occur. When such unintended non-specific hybridization occurs, it is possible that sequences other than the targeted sequence will be

10 amplified. If these limitations of PCR and LCR could be reduced or eliminated, the methods would be even more useful than they presently are.

Summary of the Invention

The invention relates to isolated nucleic acids which

15 encode a thermostable protein that enhances specific binding of a thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid. As used herein, bulge loops include mispaired bases and frameshifts of 1-4 nucleotides or more. A protein which enhances specific

20 binding of a thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid is defined herein to include proteins which increase the occurrence of binding to bulge loops in a heteroduplex nucleic acid by a thermostable mismatch binding protein and proteins which

25 increase the stability of complexes produced by binding of a thermostable mismatch binding protein to a bulge loop in a heteroduplex nucleic acid. A complex produced by binding of a thermostable mismatch binding protein to a bulge loop in a heteroduplex nucleic acid is referred to herein as a

30 "thermostable bulge loop-binding protein-heteroduplex nucleic acid complex".

In one embodiment, the invention relates to nucleic acids which encode thermostable MutL proteins. Such nucleic acids include, for example, nucleic acids encoding

Aquifex pyrophilus MutL, *Thermotoga maritima* MutL or *Thermus thermophilus* MutL, and nucleic acids which hybridize to these nucleic acids and encode a thermostable protein that enhances binding of a thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid. In another embodiment, the invention relates to nucleic acids which hybridize to nucleic acids encoding *Aquifex pyrophilus* MutL, *Thermotoga maritima* MutL or *Thermus thermophilus* MutL and are useful as probes or primers to detect and/or recover homologous genes from other hyperthermophilic or thermophilic bacteria, including homologous genes from members of the genus *Aquifex* other than *Aquifex pyrophilus*, from members of the genus *Thermotoga* other than *Thermotoga maritima* and from members of the genus *Thermus* other than *Thermus thermophilus*. The invention further relates to recombinant constructs and vectors comprising nucleic acids that encode *Aquifex pyrophilus* MutL, *Thermotoga maritima* MutL or *Thermus thermophilus* MutL, or nucleic acids which hybridize thereto.

The invention also relates to proteins isolated from hyperthermophilic and thermophilic bacteria that enhance binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid. As used herein, the phrase "isolated from" or "isolated nucleic acid" refers to nucleic acid obtained from (isolated from) naturally occurring sources as well as nucleic acids produced by recombinant methods or chemical synthesis, or by combinations of biological and chemical methods. Isolated nucleic acids produced by recombinant methods (e.g., genetic engineering methods) or synthesized chemically can also be referred to, respectively, as recombinantly produced nucleic acids and chemically synthesized or synthetic nucleic acids.

The invention further relates to isolated MutL proteins from hyperthermophilic or thermophilic bacteria. "Isolated" MutL proteins from hyperthermophilic or thermophilic bacteria include those obtained from naturally-occurring sources, as well as those produced by recombinant methods or chemical synthesis, or by combinations of biological and chemical methods.

The invention also relates to isolated thermostable proteins or polypeptides that enhance binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid. Recombinant thermostable proteins that enhance binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid can be produced in host cells using cells and methods described herein.

Another embodiment of the invention relates to a method of reducing DNA misincorporation (i.e., improving fidelity of DNA replication) in an amplification reaction by including a thermostable mismatch binding protein with a thermostable protein that enhances binding of the thermostable mismatch binding protein to bulge loops in the reaction. The thermostable mismatch binding protein binds to bulge loops in a heteroduplex nucleic acid formed as a result of misincorporation of deoxynucleoside triphosphates during the amplification reaction. This results in formation of a thermostable bulge loop-binding protein-heteroduplex nucleic acid complex. Binding of the thermostable protein prevents nucleic acids which include misincorporated deoxynucleoside triphosphates from acting as templates in subsequent rounds of the amplification reaction. Thus, amplification of nucleic acids which include misincorporated deoxynucleoside triphosphates is prevented, resulting in a reduction in overall DNA misincorporation. The thermostable protein that enhances binding of the thermostable mismatch binding protein to

bulge loops in a heteroduplex nucleic acid improves this reaction. As used herein, "thermostable bulge loop-binding protein" refers to a thermostable mismatch binding protein.

The present invention further relates to a method for
5 detecting a target nucleic acid which includes a specific
sequence comprising combining a thermostable mismatch
binding protein which binds specifically to bulge loops in
a heteroduplex nucleic acid, a thermostable protein that
enhances binding of the thermostable mismatch binding
10 protein to the bulge loops, and an amplification reaction
mixture, to produce a test combination. The individual
components of an amplification reaction mixture can each be
added, together or separately (e.g., individually), in any
order, prior to, subsequent to or simultaneously with the
15 thermostable mismatch binding protein which binds
specifically to bulge loops in a heteroduplex nucleic acid,
and/or the thermostable protein that enhances binding of
the thermostable mismatch binding protein to the bulge
loops. The resulting test combination is maintained under
20 conditions appropriate for nucleic acid amplification to
occur (i.e., synthesis of extension product). The amount
of extension product synthesized in the test combination is
determined and compared with the amount of product
synthesized in a corresponding negative control (the
25 control amount) to determine if the specific sequence
suspected of being present in the nucleic acids being
assessed is present. If the amount of product synthesized
in the test combination is the same as or less than the
amount of product synthesized in the corresponding negative
30 control, then the nucleic acids being assessed do not
include the specific sequence. If the amount of product
synthesized in the test combination is greater than the
amount of product synthesized in the corresponding control,
then the nucleic acids being assessed include the specific
35 sequence.

In one embodiment, the amplification reaction mixture comprises (1) a nucleic acid to be assessed for a specific sequence of interest; (2) four different nucleoside triphosphates; (3) two oligonucleotide primers where each primer is selected to be complementary to different strands of the nucleic acid which includes the specific sequence of interest, such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer, at a temperature which promotes hybridization of each primer to its complementary strand; (4) a blocking oligonucleotide completely complementary to the sequence of interest; (5) a thermostable enzyme which catalyzes combination of the nucleoside triphosphates to form primer extension products complementary to each strand of the nucleic acid which includes the sequence of interest; and (6) an amplification buffer suitable for the activity of the enzyme. Thus, for example, one or more of the different nucleoside triphosphates can be added prior to, subsequent to or simultaneously with the thermostable mismatch binding protein which binds specifically to bulge loops in a heteroduplex nucleic acid and/or the thermostable protein that enhances binding of the thermostable mismatch binding protein to the bulge loops. One or more of the primers can be added prior to, subsequent to or simultaneously with one or more of the different nucleoside triphosphates, the thermostable mismatch binding protein and/or the thermostable protein that enhances binding of the thermostable mismatch binding protein to the bulge loops. Similarly, the blocking oligonucleotide, the thermostable enzyme, the nucleic acid to be assessed for the specific sequence of interest and/or the amplification buffer can each be added prior to, subsequent to or simultaneously with one or more of the different nucleoside triphosphates, one or more of the

primer, the thermostable mismatch binding protein and/or the thermostable protein that enhances binding of the thermostable mismatch binding protein to the bulge loops. The blocking oligonucleotide, the thermostable enzyme, the

5 nucleic acid to be assessed for the specific sequence of interest, and the amplification buffer can also be added in any order relative to each other. As used herein, the term "blocking oligonucleotide" refers to an oligonucleotide, whether occurring naturally as in a purified restriction

10 digest or produced synthetically, which is capable of inhibiting propagation of polymerization of a primer extension product (i.e., inhibiting elongation of the extension product) when placed under conditions in which primer extension product is elongated. The blocking

15 oligonucleotide is modified at the 3' end to prevent it from functioning as a primer. Such a blocking oligonucleotide is also referred to herein as an "unextendable oligonucleotide". For example, the oligonucleotide can be modified with a 3' phosphate to

20 prevent it from functioning as a primer in the presence of Taq polymerase.

In another embodiment, the amplification reaction mixture comprises (1) a nucleic acid to be assessed for a specific sequence of interest; (2) four different

25 nucleoside triphosphates; (3) two oligonucleotide primers where each primer is selected to be complementary to different strands of the nucleic acid which includes the specific sequence of interest, with one primer completely complementary to the sequence of interest, such that the

30 extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer, at a temperature which promotes hybridization of each primer to its complementary strand; (4) a thermostable enzyme

35 which catalyzes combination of the nucleoside triphosphates

to form primer extension products complementary to each strand of the nucleic acid which includes the specific sequence of interest; and (5) an amplification buffer suitable for the activity of the enzyme. In a particular embodiment, the amplification reaction mixture further comprises a blocking oligonucleotide completely complementary to the complementary strand of the sequence of interest.

In a further embodiment, the amplification reaction mixture comprises (1) a nucleic acid to be assessed for a specific sequence of interest; (2) four oligonucleotide probes, two primary and two secondary probes, with one primary probe completely complementary to the specific sequence of interest and one secondary probe completely complementary to the complementary strand of the specific sequence of interest; (3) a thermostable enzyme which catalyzes fusion of oligonucleotide probes to form amplified products complementary to each strand of the nucleic acid which includes the specific sequence of interest; and (4) an amplification buffer suitable for the activity of the enzyme. In a particular embodiment, one of the probes which is completely complementary to the specific sequence of interest is omitted. As used herein, the term "probe" is defined to include an oligonucleotide, whether occurring naturally as in a purified restriction digest for example, or produced synthetically, which is capable of being covalently fused or ligated together into a product which is complementary to a nucleic acid strand of the target template when placed under conditions in which product formation is initiated.

As a negative control, a mixture containing (1) a nucleic acid which does not have the specific sequence thought to be included in the template being evaluated (i.e., containing only mismatched versions of the template being evaluated) and (2) the oligonucleotide designed to be

completely complementary to the specific sequence thought to be included in the template being evaluated, is maintained under (a) conditions in which primer extension is initiated in the case where the oligonucleotide is a primer or under (b) conditions in which primer extension product is elongated in the case where the oligonucleotide is a blocking oligonucleotide or under (c) conditions in which target template is amplified in the case where the oligonucleotide is a probe. The amount of amplification product synthesized in the control is compared to the amount of amplification product synthesized in a sample which comprises template nucleic acids assessed for the specific sequence of interest. If the amount of amplification product synthesized in the sample which comprises template nucleic acids assessed for the specific sequence of interest is the same as or less than the amount of amplification product synthesized in the control, the specific sequence of interest is likely not included in the template nucleic acid. In the case of the opposite result (if the amount of amplification product synthesized in the sample which comprises template nucleic acids assessed for the specific sequence of interest is greater than the amount of amplification product synthesized in the control), the specific sequence of interest is likely included in the template nucleic acid.

In a particular embodiment, the specific sequence of interest is a mutation.

The present invention also relates to a method for amplifying a nucleic acid comprising a specific sequence of interest. The method comprises (a) combining a thermostable mismatch binding protein which binds specifically to bulge loops in a heteroduplex nucleic acid, and a thermostable protein that enhances binding of the thermostable mismatch binding protein to bulge loops and an amplification reaction mixture, thereby producing a test

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combination; and (b) maintaining the test combination of step (a) under conditions appropriate for amplification of nucleic acids to occur, resulting in synthesis of the nucleic acid comprising the sequence of interest. In a particular embodiment, the amplification reaction mixture includes (1) a nucleic acid comprising a specific sequence to be amplified; (2) four different nucleoside triphosphates; (3) two oligonucleotide primers where each primer is selected to be completely complementary to different strands of the nucleic acid comprising the specific sequence to be amplified; (4) blocking oligonucleotides which form heteroduplexes with a strand of the nucleic acids being selected against; (5) a thermostable enzyme which catalyzes combination of the nucleoside triphosphates to form primer extension products complementary to each strand of the nucleic acid comprising the specific sequence to be amplified; and (6) an amplification buffer suitable for the activity of the enzyme. The individual components of the amplification reaction mixture can each be added, together or individually and separately in any order, prior to, subsequent to or simultaneously with the thermostable mismatch binding protein which binds specifically to bulge loops in a heteroduplex nucleic acid and/or the thermostable protein that enhances binding of the thermostable mismatch binding protein to the bulge loops.

The invention further relates to a method for selecting against (i.e., reducing or preventing amplification of) a nucleic acid comprising a specific sequence of interest. The method comprises (a) combining a thermostable mismatch binding protein which binds specifically to bulge loops in a heteroduplex nucleic acid, a thermostable protein that enhances binding of the thermostable mismatch binding protein to bulge loops, and an amplification reaction mixture, thereby producing a test

combination and (b) maintaining the test combination of step (a) under conditions appropriate for amplification of nucleic acids to occur. The thermostable mismatch binding protein binds heteroduplexes containing the nucleic acids to be selected against, preventing them from acting as templates in subsequent rounds of the amplification reaction and thereby selecting against a nucleic acid comprising the specific sequence. The thermostable protein which enhances binding of the thermostable mismatch binding protein to bulge loops improves this reaction. In a particular embodiment, the amplification reaction mixture comprises (1) nucleic acids comprising a specific sequence to be amplified or detected and nucleic acids whose synthesis is to be prevented or reduced (nucleic acids to be selected against); (2) four different nucleoside triphosphates; (3) two oligonucleotide primers where each primer is selected to be complementary to different strands of the nucleic acid comprising the specific sequence to be amplified or detected; (4) blocking oligonucleotides which form heteroduplexes with a strand of the nucleic acid whose synthesis is to be prevented or reduced (the nucleic acid being selected against); (5) a thermostable enzyme which catalyzes combination of the nucleoside triphosphates to form primer extension products complementary to each strand of the nucleic acid comprising the specific sequence to be amplified or detected; and (6) an amplification buffer suitable for the activity of the enzyme. The individual components of the amplification reaction mixture can each be added, together or separately (e.g., individually) in any order, prior to, subsequent to or simultaneously with the thermostable mismatch binding protein which binds specifically to bulge loops in a heteroduplex nucleic acid and/or the thermostable protein that enhances binding of the thermostable mismatch binding protein to the bulge loops.

In each particular embodiment, the amplification reaction mixture can further include additional components, such as, for example, components which enhance the activity of thermostable enzymes to catalyze combination of
5 nucleoside triphosphates to form primer extension products or components which enhance and/or improve the amplification reaction and/or the utility of the amplification procedure.

The invention further relates to an improvement in a
10 method of amplification wherein the improvement comprises adding a thermostable protein that enhances specific binding of a thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid to a solution comprising an amplification reaction mixture and the
15 thermostable mismatch binding protein. Thermostable MutL protein is an example of a thermostable protein that enhances specific binding of a thermostable mismatch binding protein to bulge loops that can be added.

The methods of the invention can further comprise
20 including a stabilizer. As used herein, a stabilizer increases the lifetime of a thermostable bulge loop-binding protein-heteroduplex nucleic acid complex. A thermostable bulge loop-binding-heteroduplex nucleic acid complex is a complex formed when the thermostable mismatch binding
25 protein is bound to a bulge loop in a heteroduplex nucleic acid. ATP γ S is an example of a stabilizer.

Oligonucleotides which are designed so that they form heteroduplexes with a strand of the nucleic acid differ at one or more base pairs, at one or more sites, from the
30 nucleic acid to be selected against. Oligonucleotides which are designed to be completely complementary to a specific sequence of interest or are designed to form heteroduplexes with a strand of the nucleic acid can be primers, blocking oligonucleotides or probes.

The components of an amplification reaction mixture and amplification conditions depend upon the particular amplification procedure being employed and can be determined from readily available sources. The components of an amplification mixture further depend on whether the specific sequence of interest is in, for example, a region of high GC content or a region of high AT content. Amplification procedures include, for example, PCR, LCR and their variations.

10 Brief Description of the Drawings

Figure 1 depicts the DNA sequence (SEQ ID NO:1) of the coding region of *Aquifex pyrophilus* (Apy) MutS.

Figure 2 depicts the amino acid sequence (SEQ ID NO:2) of *Aquifex pyrophilus* MutS.

15 Figure 3 depicts the DNA sequence (SEQ ID NO:4) of the coding region of *Thermotoga maritima* (Tma) MutS.

Figure 4 depicts the amino acid sequence (SEQ ID NO:5) of *Thermotoga maritima* MutS.

20 Figure 5 depicts the partial DNA sequence (SEQ ID NO:6) of the coding region of *Thermus thermophilus* MutS.

Figure 6 depicts the partial DNA sequence (SEQ ID NO:7) of the coding region of *Thermus aquaticus* MutS.

Figure 7 depicts the alignment of partial amino acid sequences for the coding regions of *Aquifex pyrophilus* MutS (SEQ ID NO:2), *Thermus aquaticus* (Taq) MutS (SEQ ID NO:8), *Thermus thermophilus* (Tth) MutS (SEQ ID NO:9) and *Thermotoga maritima* MutS (SEQ ID NO:5). The numbers "613" and "595" correspond to amino acid position 613 in Apy MutS and amino acid position 595 in Tma MutS, respectively.

30 Figure 8 depicts the DNA sequence (SEQ ID NO:39) of the coding region of *Aquifex pyrophilus* MutL.

Figure 9 depicts the DNA sequence (SEQ ID NO:41) of the coding region of *Thermotoga maritima* MutL.

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Figure 10 depicts the amino acid sequences of *Escherichia (E.) coli* (Eco) MutS (SEQ ID NO:3), *Aquifex (A.) pyrophilus* MutS (SEQ ID NO:2) and *Thermotoga (T.) maritima* MutS (SEQ ID NO:5), with (|) indicating identical amino acids and (:) indicating similar amino acids (TFASTA).

Figure 11 depicts the amino acid sequences of *Aquifex pyrophilus* (Apy) MutL (SEQ ID NO:40), *Thermotoga maritima* (Tma) MutL (SEQ ID NO:42), *Streptococcus (S.) pneumoniae* (Spn) HexB (SEQ ID NO:43) and *Escherichia (E.) coli* (Eco) MutL (SEQ ID NO:44) (PILEUP).

Figure 12 depicts an analysis of the 5' and 3' untranslated regions of Tma MutS. Initiation : Double underlines indicate, in order, an in frame termination codon (TGA), a valine codon (GTN), a termination codon (TGA) for an upstream open reading frame (orf), the region of similarity to the 3' end of Tma 16S rRNA, and two additional valine codons. Termination : Double underlines indicate the antisense termination codon (TCA) for a downstream, antisense open reading frame (orf) and the termination codon (TGA) for Tma MutS. Proteins with identical (|) or similar (:) amino acids (TFASTA) to the open reading frame are shown.

Figure 13 depicts the partial DNA sequence (SEQ ID NO:45) of the coding region of *Thermus thermophilus* MutL.

Figure 14 depicts the alignment of partial amino acid sequences for the coding regions of *E. coli* MutL (SEQ ID NO:44), *Thermus thermophilus* MutL (SEQ ID NO:45) and *S. pneumoniae* HexB (SEQ ID NO:43). The numbers refer to the positions of the amino acids in *E. coli* MutL.

Detailed Description of the Invention

Mismatch correction in prokaryotic and eukaryotic species may be initiated by the mismatch binding of a homolog of the product of one of several *E. coli* mutator

genes, *mutS*. In *E. coli*, mismatch correction also requires MutL, the endonucleolytic activity of MutH, and the activities of several additional enzymes (Modrich, P., *Annu. Rev. Genet.* 25: 229-253 (1991); Modrich, P., *Science* 5 266: 1959-1960 (1994)). Insertions into *mutS* lead to a high frequency of spontaneous mutation which may easily be detected as an increased frequency of streptomycin resistant cells (Siegel, E.C. et al., *Mutat. Res.* 93: 25-33 (1982)). The MutHSL system selectively removes mismatches 10 from daughter strands following incorrect incorporation of nucleotides during DNA replication (Au, K.G. et al., *J. Biol. Chem.* 267: 12142-12148 (1992)). In *E. coli*, GATC sites are methylated by the *dam* methylase. Hemimethylation at GATC permits differentiation of template from daughter 15 strands. The repair of a mismatch is bidirectional with respect to the hemimethylated site (Cooper, D.L. et al., *J. Biol. Chem.* 268: 11823-11829 (1993)). In addition, the same mismatch correction system is responsible for removing frameshifts of up to four nucleotides which may be the 20 result of the presence of an intercalating agent during DNA replication (Rene, B. et al., *Mutat. Res.* 193: 269-273 (1988)) or of polymerase slippage at di- or tri-nucleotide repeats (Parker, B.O. and Marinus, M.G., *Proc. Natl. Acad. Sci. USA* 89: 1730-1734 (1992)). Transition and frameshift 25 mutations are increased about 275- and 1500-fold, respectively, in *mutS*⁻ *E. coli* cells (Schaaper, R.M. and Dunn, R.L., *Genetics* 129: 317-326 (1991)).

In man, the *mutS* homolog (*MSH2*) is a mutator gene involved in hereditary nonpolyposis colorectal cancer 30 (Leach, F.S. et al., *Cell* 75: 1215-1225 (1993); Fishel, R. et al., *Cell* 75: 1027-1038 (1993)), and there are now phenotypes for a growing list of human mismatch repair proteins. Cells deficient in MutS homolog-dependent mismatch repair fail to accumulate single-strand breaks and 35 are resistant to killing by alkylating agents (Branch, P.

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et al., *Nature* 362: 652-654 (1993)), suggesting that in wild-type cells, introduction of alkylated sites reactivates mismatch repair and that MutS homologs find target sites, whether they be mismatches or other small lesions. In fact, the replication of alkylated DNA in *mutS*⁻ *E. coli* cells may contribute to the hypermutation phenotype.

Purified *E. coli* MutS protein binds specifically to oligonucleotide heteroduplexes (Su, S.-S. and Modrich, P., *Proc. Natl. Acad. Sci. USA* 83: 5057-5061 (1985)). Gel-shift assays may be carried-out with *E. coli* MutS protein and a heteroduplex with a GT mismatch (less efficiently an AC mismatch) (Jiricny, J. et al., *Nucleic Acids Res.* 16: 7843-7853 (1988)) or a 3-nucleotide bulge loop (Lishanski, A. et al., *Proc. Natl. Acad. Sci. USA* 91: 2674-2678 (1994)) to detect MutS protein binding. *E. coli* MutS protein also binds specifically to heteroduplexes containing IC mismatches (Jiricny, J. et al., *Nucleic Acids Res.* 16: 7843-7853 (1988)). Human MSH2 also binds to GT mismatches (Fishel, R. et al., *Cancer Res.* 54: 5539-5542 (1994)). However, binding to bulge loops is not limited to 1-4 nucleotides but occurs with loops as large as 14 nucleotides in length (Fishel, R. et al., *Science* 266: 1403-1405 (1994)). The binding of *E. coli* MutS protein to mismatches in the presence of *E. coli* MutL protein is sufficiently strong that it will block RecA-mediated strand displacement reactions (Worth, L., Jr. et al., *Proc. Natl. Acad. Sci. USA* 91: 3238-3241 (1994)) and by itself the exonuclease activity of T7 DNA polymerase (Ellis, L.A. et al., *Nucleic Acids Res.* 22: 2710-2711 (1994)).

Applicant has cloned and expressed thermostable MutL proteins from hyperthermophilic eubacteria and demonstrated that specific binding of thermostable MutS proteins to bulge loops in a heteroduplex nucleic acid is enhanced in the presence of a thermostable MutL protein. Until

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Applicant's cloning and isolation of thermostable MutL proteins, all of the studies of MutL and MutL-homolog proteins have involved proteins from mesophilic organisms.

As used herein, the term "thermostable protein" refers to protein of thermophilic bacterial origin or hyperthermophilic bacterial origin. Such thermostable proteins can be obtained from an organism in which they occur in nature, can be produced by recombinant methods or can be synthesized chemically.

As used herein, the terms "heteroduplex nucleic acid" and "heteroduplex" refer to a double-stranded nucleic acid which is formed by a mismatch (e.g., C-A or G-T nucleotide pairs as opposed to the naturally-occurring C-G or A-T nucleotide pairs or frameshifts of 1-4 nucleotides or more) between complementary strands. As used herein, the terms "homoduplex nucleic acid" and "homoduplex" refer to a double-stranded nucleic acid which is formed by perfectly matched complementary strands. As defined herein, a bulge loop is a distortion in double-stranded nucleic acids. A bulge loop arises as a result of, for example, a frameshift or a mispairing between strands in a limited region, i.e., a mismatch between complementary strands, and comprises a mismatch of at least a single nucleotide.

Nucleic Acids, Constructs and Vectors

The present invention relates to isolated nucleic acids which encode a thermostable protein that enhances specific binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid. A protein which enhances specific binding of a thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid is defined herein to include proteins which increase the occurrence of binding to bulge loops in a heteroduplex nucleic acid by a thermostable mismatch binding protein and proteins which increase the stability of complexes produced

by binding of a thermostable mismatch binding protein to a bulge loop in a heteroduplex nucleic acid. A complex produced by binding of a thermostable mismatch binding protein to a bulge loop in a heteroduplex nucleic acid is referred to herein as a "thermostable bulge loop-binding protein-heteroduplex nucleic acid complex". As used herein, "thermostable mismatch binding proteins" are proteins, polypeptides or protein fragments which are stable to heat, bind specifically to bulge loops in a heteroduplex nucleic acid, have heat resistant nucleic acid binding activity and do not become irreversibly denatured (inactivated) when subjected to the elevated temperatures for the time periods necessary, for example, for PCR amplification. Examples of thermostable mismatch binding proteins include thermostable MutS proteins from *Aquifex pyrophilus*, *Thermotoga maritima*, *Thermus thermophilus* and *Thermus aquaticus*, and variants (e.g. mutants) of those proteins and/or portions thereof. Thermostable MutS proteins and methods for their production are described herein, and in U.S. Application No. 08/468,558 (filed June 6, 1995) and International Application No. PCT/US96/08677 (filed June 4, 1996). See also International Publication No. WO 96/39525 (published December 12, 1996). A thermostable MutS protein from *Thermus aquaticus* is described by Biswas, I. and Hsieh, P. (*J. Biol. Chem.* 271(9):5040-5048 (1996)). A thermostable MutS protein from *Thermus thermophilus* is described by Takamatsu, S. et al. (*Nucleic Acids Research* 24(4):640-647 (1996)).

In one embodiment, the nucleic acid encodes a thermostable protein that enhances specific binding of thermostable MutS proteins to bulge loops in a heteroduplex nucleic acid. The present invention also relates more specifically to isolated nucleic acids which encode a thermostable MutL protein from hyperthermophilic or

thermophilic bacteria. The present invention further relates to isolated nucleic acids which encode a thermostable MutL protein from *Aquifex pyrophilus* and isolated nucleic acids which encode a thermostable MutL protein from *Thermotoga maritima*. The present invention also relates to isolated nucleic acids which encode a thermostable MutL protein from *Thermus thermophilus*.

The invention also relates to isolated nucleic acids which (1) hybridize to (a) a nucleic acid encoding a thermostable MutL protein, such as a nucleic acid having the sequence of Figure 8 (SEQ ID NO:39), Figure 9 (SEQ ID NO:41) or Figure 13 (SEQ ID NO:45), (b) the complement of any one of (a), or (c) portions of either of the foregoing (e.g., a portion comprising the open reading frame); (2) encode a polypeptide having the amino acid sequence of a thermostable MutL protein (e.g., SEQ ID NO:40 or SEQ ID NO:42), or functional equivalents thereof (e.g., a thermostable polypeptide that enhances specific binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid with a selected amino acid); or (3) have both characteristics. Portions of the isolated nucleic acids which code for polypeptides having a certain function can be identified and isolated by, for example, the method of Jasin, M., et al., U.S. Patent No. 4,952,501.

Nucleic acids meeting these criteria comprise nucleic acids having sequences identical to sequences of naturally occurring thermostable MutL proteins from *Aquifex pyrophilus*, *Thermotoga maritima* or *Thermus thermophilus*, or variants of the naturally occurring sequences. Such variants include mutants differing from naturally occurring sequences by the addition, deletion or substitution of one or more residues, modified nucleic acids in which one or more residues are modified (e.g., DNA or RNA analogs), and mutants comprising one or more modified residues.

Such nucleic acids can be detected and isolated under high stringency conditions or moderate stringency conditions, for example. "High stringency conditions" and "moderate stringency conditions" for nucleic acid hybridizations are set forth on pages 2.10.1-2.10.16 (see particularly 2.10.8-11) and pages 6.3.1-6 in *Current Protocols in Molecular Biology* (Ausubel, F.M. et al., Vol. 1, Suppl. 26, 1991). Factors such as probe length, base composition, percent mismatch between the hybridizing sequences, temperature and ionic strength influence the stability of nucleic acid hybrids. Thus, high or moderate stringency conditions can be determined empirically, depending in part upon the characteristics of the known DNA to which other unknown nucleic acids are being compared for sequence similarity.

Isolated nucleic acids that are characterized by their ability to hybridize to (a) a nucleic acid encoding a thermostable MutL protein (for example, those nucleic acids depicted in Figure 8 (SEQ ID NO:39), Figure 9 (SEQ ID NO:41) and Figure 13 (SEQ ID NO:45), (b) the complement of such nucleic acids, (c) or a portion thereof (e.g. under high or moderate stringency conditions), and which encode a thermostable protein or polypeptide which enhances specific binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid are also the subject of this invention. The binding function of a protein or polypeptide encoded by hybridizing nucleic acid may be detected by standard assays for binding (e.g., mismatch binding assays which demonstrate binding of the protein or polypeptide to a bulge loop in a heteroduplex nucleic acid such as, for example, gel shift assays). Functions characteristic of the thermostable MutL protein may also be assessed by *in vivo* complementation tests or other suitable methods. Mismatch binding assays, complementation tests, or other suitable methods can also be used in procedures

for the identification and/or isolation of nucleic acids which encode a polypeptide such as a polypeptide of the amino acid sequence SEQ ID NO:40 or SEQ ID NO:42, or functional equivalents of these polypeptides. The
5 antigenic properties of proteins or polypeptides encoded by hybridizing nucleic acids can be determined by immunological methods employing antibodies that also bind to a naturally-occurring thermostable MutL protein. These methods can include immunoblot, immunoprecipitation and
10 radioimmunoassay.

Nucleic acids of the present invention can be used in the production of proteins or polypeptides. For example, DNA encoding a thermostable MutL protein, such as a thermostable MutL from *Aquifex pyrophilus*, or DNA which
15 hybridizes to DNA having the sequence SEQ ID NO:39, can be incorporated into various constructs and vectors created for further manipulation of sequences or for production of the encoded polypeptide in suitable host cells. Similarly, DNA containing all or part of the coding sequence for a
20 thermostable MutL protein, such as a thermostable MutL from *Thermotoga maritima*, or DNA which hybridizes to DNA having the sequence SEQ ID NO:41, can be incorporated into various constructs and vectors created for further manipulation of sequences or for production of the encoded polypeptide in
25 suitable host cells. For expression in *E. coli* and other organisms, a GTG initiation codon can be altered to ATG as appropriate.

Nucleic acids referred to herein as "isolated" are nucleic acids separated away from the nucleic acids of the
30 genomic DNA or cellular RNA of their source of origin (e.g., as it exists in cells or in a mixture of nucleic acids such as a library), and may have undergone further processing. "Isolated" nucleic acids include nucleic acids obtained by methods described herein, similar methods or
35 other suitable methods, including essentially pure nucleic

acids, nucleic acids produced by chemical synthesis, by combinations of biological and chemical methods, and recombinant nucleic acids which are isolated. Nucleic acids referred to herein as "recombinant" are nucleic acids

5 which have been produced by recombinant DNA methodology, including those nucleic acids that are generated by procedures which rely upon a method of artificial recombination, such as the polymerase chain reaction (PCR) and/or cloning into a vector using restriction enzymes.

10 "Recombinant" nucleic acids are also those that result from recombination events that occur through the natural mechanisms of cells, but are selected for after the introduction to the cells of nucleic acids designed to allow and make probable a desired recombination event.

15 MutL proteins from hyperthermophiles such as *Aquifex pyrophilus*, *Thermotoga maritima* and *Thermus thermophilus* can be used in methods for allele-specific amplification and in methods for enhancing amplification reactions because they are stable to heat, are heat resistant and do

20 not become irreversibly denatured (inactivated) when subjected to the elevated temperatures for the length of time necessary for the denaturation and annealing steps of amplification techniques such as the polymerase chain reaction and its variations or the ligase chain reaction

25 and its variations.

As described in the Examples, MutL genes were cloned into *E. coli* from two distantly-related hyperthermophilic eubacteria, *Aquifex pyrophilus* (Apy) and *Thermotoga maritima* (Tma). All cloning was carried out using PCR

30 technology without the need for library construction. Inverse PCR is a rapid method for obtaining sequence data for the 5'- and 3'-flanking regions of bacterial genes, the prerequisite for generation of primers for PCR cloning into an expression vector. Because of the inherent error

35 frequency of *in vitro* DNA replication, care was taken to

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demonstrate that sequences of independently-derived expression clones were identical. A MutL protein from each species was expressed and purified to homogeneity. The proteins were thermoresistent to $\geq 90^{\circ}\text{C}$ and enhanced binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid.

The approaches described herein, including, but not limited to, the approaches to isolate and manipulate the MutL genes of *Aquifex pyrophilus* and *Thermotoga maritima*, to construct vectors and host strains, and to produce and use the proteins, to produce antibodies, etc., can be applied to other members of the genus *Aquifex* or other members of the genus *Thermotoga*. For example, the Apy MutL gene described here, or sufficient portions thereof, including fragments produced by PCR, can be used as probes or primers to detect and/or recover homologous genes of the other *Aquifex* species (e.g., by hybridization, PCR or other suitable techniques). Similarly, genes encoding Apy MutL and other *Aquifex* species MutL proteins can be isolated from genomic libraries according to methods described herein or other suitable methods. The Tma MutL gene described here, or sufficient portions thereof, including fragments produced by PCR, can be used as probes or primers to detect and/or recover homologous genes of the other *Thermotoga* species (e.g., by hybridization, PCR or other suitable techniques). Similarly, genes encoding Tma MutL and other *Thermotoga* species MutL proteins can be isolated from genomic libraries according to methods described herein or other suitable methods. *Aquifex* and *Thermotoga* species MutL proteins can be evaluated for their ability to enhance binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid using methods described herein for evaluating the ability of Apy and Tma MutL proteins to enhance binding of thermostable mismatch

binding proteins to bulge loops in a heteroduplex nucleic acid (e.g., gel shift binding assays).

The approaches described herein, including, but not limited to, the approaches to isolate and manipulate the MutL genes of *Aquifex pyrophilus* and *Thermotoga maritima*, to construct vectors and host strains, and to produce and use the proteins, to produce antibodies, etc., can also be applied to other hyperthermophilic bacteria and to thermophilic bacteria. Hyperthermophilic bacteria include species of the archaeobacteria, which include the most hyperthermophilic species known. Hyperthermophilic archaeobacteria include members of the genus *Pyrodictium*, including, but not limited to, *Pyrodictium abyssi* (Pab) and *Pyrodictium occultum* (Poc). Thermophilic bacteria include members of the genus *Thermus*, including, but not limited to, *Thermus aquaticus* (Taq) and *Thermus thermophilus* (Tth). Thermophilic bacteria also include hyperthermophilic bacteria. As used herein, "thermophilic bacteria" is meant to include hyperthermophilic and thermophilic bacteria.

For example, the partial DNA sequence (SEQ ID NO:45) of the coding region of Tth MutL protein was isolated according to methods described herein to isolate and manipulate the MutL genes of *Aquifex pyrophilus* and *Thermotoga maritima*. The partial Tth MutL DNA sequence described herein, or sufficient portions thereof, including fragments produced by PCR, can be used as probes or primers to detect and/or recover homologous DNA sequences and/or genes of the other *Thermus* species (e.g., by hybridization, PCR or other suitable techniques). Genomic DNA from several *Thermus* species (e.g., *Thermus thermophilus* and *Thermus aquaticus*) can be obtained, for example, from the American Type Culture Collection.

Hyperthermophilic archaeobacteria *Pyrodictium abyssi* and *Pyrodictium occultum*, both from cells supplied by Professor Karl Stetter, Universität Regensburg, can be used

as templates for degenerate priming. Once Pab and Poc fragment sequences have been found which encode an amino acid sequence similar to other MutL proteins, unique inverse primers can be synthesized and tested by Southern hybridization to verify that these sequences originated from Pab and Poc genomic DNAs.

The 5' coding and 3' downstream noncoding sequences for Pab, Poc and *Thermus* species (e.g., Taq and Tth) *mutL* can be obtained by inverse PCR walking. The 5' coding sequence can be verified by cycle sequencing. These coding sequences can be used to design expression primers. Independently-derived PCR products resulting from each pair of expression primers can be ligated into one or more expression plasmids, including pDG160/pDG182/pDG184 and/or the pET series from Novagen, Inc., and electroporated into the appropriate hosts. Plasmids from several clones expressing each thermostable MutL can be sequenced.

The PCR amplifications of Pab, Poc and *Thermus* species genomic DNAs can be carried out in 50-100 μ l containing 1 μ M of each primer, 10 mM Tris buffer, pH 8.3, 50 mM KCl, 25-50 units/ml Taq DNA polymerase, and 200 μ M of each dNTP (Saiki, R.K. et al., *Science* 239: 487-491 (1988)). Simultaneous reactions can be initiated by addition of a $MgCl_2$ solution to Mg^{++} -free PCR mixtures at $>80^\circ C$ to yield final concentrations of 0.8-2 mM followed by denaturation for 30 seconds at $95^\circ C$.

When using degenerate primers and 50 ng of a genomic DNA template, the first 5 cycles will employ a 30 second annealing step at $45^\circ C$ followed by a 2 minute ramp to $72^\circ C$ before denaturation. An additional 30-35 cycles can be carried out with a $55^\circ C$ annealing temperature. For inverse PCR (Ochman, H. et al., In PCR Protocols. A Guide to Methods and Applications, Innis, M.A. et al., Eds. (San Diego: Academic Press, Inc.) pp. 219-227 (1990)), genomic DNA can be digested to completion with a restriction

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endonuclease leaving a 3' or 5' 4-base overhang, phenol extracted, and ligated overnight at a DNA concentration of less than 50 µg/ml. When using unique direct or inverse PCR primers, 50 ng of genomic or circularized genomic DNA template, respectively, can be employed, and the first 5 cycles omitted.

Thermostable protein mixtures from bacteria expressing Pab, Poc or a *Thermus* species MutL can be prepared and purified as described in the Examples pertaining to the preparation and purification of Apy and Tma MutL. The purification scheme can be optimized for each protein using routine experimentation. The proteins can be concentrated, and the solvent can be changed by dialysis. The final products can be analyzed for purity by SDS-PAGE. Protein concentrations can be determined using the Bio-Rad Protein Assay kit (Bradford) and by analysis of complete absorbance spectra, which will document removal of nucleic acids.

These purified MutL proteins can be evaluated for the ability to enhance binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid using the methods described herein in evaluating the ability of the Apy and Tma MutL proteins to enhance binding of Apy and Tma MutS proteins to a bulge loop in a heteroduplex nucleic acid (see, e.g., gel shift assays).

25 Proteins

The invention also relates to thermostable proteins or polypeptides encoded by nucleic acids of the present invention. The thermostable proteins and polypeptides of the present invention enhance specific binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid. As used herein, "thermostable proteins or polypeptides" are proteins, polypeptides or protein fragments which are stable to heat, have heat resistant activity (e.g., the ability to enhance specific

binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid), and do not become irreversibly denatured (inactivated) when subjected to the elevated temperatures for the time periods necessary, for example, for PCR amplification. Thermostable proteins are also proteins of thermophilic bacterial origin or hyperthermophilic bacterial origin. Such proteins can be obtained from (isolated from) an organism in which they occur in nature, can be produced by recombinant methods or can be synthesized chemically.

The thermostable proteins described herein are thermoresistant to $\geq 90^{\circ}\text{C}$. The thermostable proteins are known to enhance specific binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid at temperatures of from about room temperature to about 90°C . However, specificity of binding to bulge loops is greatest at the high end of this temperature range. With decreasing temperature from about 60°C , an increasing proportion of protein is found to bind nonspecifically to nucleic acids forming perfect homoduplexes.

The thermostable proteins and polypeptides of the present invention can be isolated and/or recombinant. Proteins or polypeptides referred to herein as "isolated" are proteins or polypeptides purified to a state beyond that in which they exist in cells. "Isolated" proteins or polypeptides include proteins or polypeptides obtained by methods described herein, similar methods or other suitable methods, including essentially pure proteins or polypeptides, proteins or polypeptides produced by chemical synthesis, by recombinant methods, or by combinations of biological and chemical methods, and recombinant proteins or polypeptides which are isolated. Proteins or polypeptides referred to herein as "recombinant" or "recombinantly produced" are proteins or polypeptides produced by the expression of recombinant nucleic acids.

In one embodiment, the thermostable protein enhances specific binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid. These thermostable proteins include, for example, naturally occurring thermostable MutL proteins from *Aquifex pyrophilus*, *Thermotoga maritima* and *Thermus thermophilus*, variants (e.g. mutants) of those proteins and/or portions thereof. Thermostable mismatch binding proteins include, for example, thermostable MutS proteins from naturally occurring, isolated and recombinant *Aquifex pyrophilus*, *Thermotoga maritima*, *Thermus thermophilus* and *Thermus aquaticus*, variants (e.g. mutants) of those proteins and/or portions thereof. As used herein, "variants" include mutants differing by the addition, deletion or substitution of one or more amino acid residues, or modified polypeptides in which one or more residues are modified, and mutants comprising one or more modified residues.

In another embodiment, like naturally occurring thermostable MutL proteins from *Aquifex pyrophilus*, *Thermotoga maritima* and *Thermus thermophilus*, isolated and/or recombinant thermostable MutL proteins of the present invention enhance specific binding of thermostable mismatch binding proteins to bulge loops in heteroduplex nucleic acids. For example, in the case of *Aquifex pyrophilus*, an isolated, recombinant thermostable MutL enhances specific binding of thermostable MutS proteins to bulge loops in a heteroduplex nucleic acid.

The invention further relates to fusion proteins, comprising a thermostable MutL protein (as described above) as a first moiety, linked to second moiety not occurring in the thermostable MutL protein as found in nature. The second moiety can be an amino acid or polypeptide. The first moiety can be in an N-terminal location, C-terminal location or internal to the fusion protein. In one embodiment, the fusion protein comprises a thermostable

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MutL protein of *Aquifex pyrophilus* origin as the first moiety, and a second moiety comprising a linker sequence and affinity ligand.

Fusion proteins can be produced by a variety of methods. For example, a fusion protein can be produced by the insertion of a thermostable MutL gene or portion thereof into a suitable expression vector, such as Bluescript SK +/- (Stratagene), pGEX-4T-2 (Pharmacia) and pET-15b (Novagen). The resulting construct is then introduced into a suitable host cell for expression. Upon expression, fusion protein can be purified from a cell lysate by means of a suitable affinity matrix (see e.g., Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, Vol. 2, Suppl. 26, pp. 16.4.1-16.7.8, 1991).

Method of Producing Recombinant Thermostable MutL Proteins

Another aspect of the invention relates to a method of producing a thermostable MutL protein, and to expression systems and host cells containing a vector appropriate for expression of a thermostable MutL protein.

Cells that express a recombinant thermostable MutL protein can be made and maintained in culture to produce protein for isolation and purification. These cells can be procaryotic or eucaryotic. Examples of procaryotic cells that can be used to express thermostable MutL proteins include *Escherichia coli*, *Bacillus subtilis* and other bacteria. Examples of eucaryotic cells that can be used to express the thermostable MutL protein include yeasts such as *Saccharomyces (S.) cerevisiae*, *S. pombe*, *Pichia pastoris*, and other lower eucaryotic cells, as well as cells of higher eucaryotes, such as those from insects and mammals. (See, e.g., Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, John Wiley & Sons Inc., New York, 1994).

To make host cells that produce a thermostable MutL protein for isolation and purification, as a first step the gene encoding the MutL protein can be inserted into a nucleic acid vector, e.g., a DNA vector, such as a plasmid, virus or other suitable replicon, which can be present in a single copy or multiple copies, or the gene can be integrated in a host cell chromosome. Such a suitable replicon contains all or part of the coding sequence for thermostable MutL protein operably linked to one or more expression control sequences whereby the coding sequence is under the control of transcription signals and linked to appropriate translation signals to permit translation of the thermostable MutL protein or of a fusion protein comprising a thermostable MutL protein. As a second step, the vector can be introduced into cells by a method appropriate to the type of host cells (e.g., transformation, transfection, electroporation, infection). In a third step, for expression from the thermostable MutL gene, the host cells can be maintained under appropriate conditions (e.g., in the presence of inducer, normal growth conditions) for expression of the gene and production of the encoded MutL protein.

As a particular example of the above approach to producing active thermostable MutL protein, a gene encoding the *Aquifex pyrophilus* MutL can be integrated into the genome of a virus that enters host cells. By infection of the host cells, the components of a system which permits the transcription and translation of the *Aquifex pyrophilus* MutL gene are introduced into the host cells, in which expression of the encoded product occurs. Alternatively, an RNA polymerase gene, inducer, or other component required to complete such a gene expression system may be introduced into the host cells already containing the *Aquifex pyrophilus* MutL gene, for example, by means of a virus that enters the host cells and contains the required

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component. The thermostable MutL gene can be under the control of an inducible or constitutive promoter. The promoter can be one that is recognized by the host cell RNA polymerase. The promoter can, alternatively, be one that is recognized by a viral RNA polymerase and is transcribed following infection of the host cells with a virus.

Mutation or Polymorphism Detection

Genome mismatch scanning (GMS) (Brown, P.O., *Current Opinion in Genetics & Development* 4: 366-373 (1994)), a method for whole genome scanning which utilizes *E. coli* MutS and the other enzymes of the mismatch repair system, is one of the new methods being developed for mapping and/or cloning genes based on sequence differences or similarities in two DNA pools (Jonsson, J.J. and Weissman, S.M., *Proc. Natl. Acad. Sci. USA* 92: 83-95 (1995)). If the gene is known, several methods have been developed for scanning the specific DNA sequences for mutations or polymorphisms, including single-strand conformation polymorphism analysis (SSCP) (reviewed by Hayashi, K. and Yandell, D.W., *Human Mutation* 2: 338-346 (1993)), which does not require heteroduplex formation, and chemical and, most recently, endonuclease VII-based cleavage methods, which require heteroduplex formation (Youil, R. et al., *Proc. Natl. Acad. Sci. USA* 92: 87-91 (1995)).

If the mutation or polymorphism is known, several methods are available for identification of specific alleles which rely on identification of internal target sequences following PCR, including allele-specific oligonucleotide hybridization (Saiki, R. K. et al., *Proc. Natl. Acad. Sci. U.S.A.* 86: 6230-6234 (1989)), oligonucleotide ligation assay (Nickerson, D.A. et al., *Proc. Natl. Acad. Sci. U.S.A.* 87: 8923-8927 (1990)) and TaqMan (Livak, K. et al., *Nat. Genet.* 9: 341-342 (1995)). The problem is relatively straightforward for mapping

germline genes, somewhat more difficult for detecting cancer-related mutations in tumors with mixed cell populations and quite difficult for screening lymph nodes or other sources (e.g. sputum) for cancer-related mutations. There are comparable problems in the analysis of mutations in pathogens. The methods for identification of specific alleles include allele-specific PCR (Kwok, S. et al., *Nucleic Acids Res.* 18: 999-1005 (1990); Tada, M. et al., *Cancer Res.* 53: 2472-2474 (1993); Bottema, C.D. et al., *Methods Enzymol.* 218: 388-402 (1993)), allele-specific ligase chain reaction (LCR) (Wiedmann, M. et al., *PCR Methods & Applications* 3: S51-64 (1994)), RFLP/PCR (Felley-Bosco, E. et al., *Nucleic Acids Res.* 19: 2913-2919 (1991); Cha, R.S. et al., *PCR. Methods. Appl.* 2: 14-20 (1992)), which requires a restriction endonuclease cleavage site in one allele, and combination methods (Hruban, R.H. et al., *Am. J. Pathol.* 143: 545-554 (1993)). *Ras* oncogene mutations have been detected by a hybridization technique subsequent to non-specific PCR in stool from patients with colorectal tumors (Sidransky, D. et al., *Science* 256: 102-105 (1992)). Mismatch-specific single-strand cleavage including MutY (Hsu, I.-C. et al., *Carcinogenesis* 15: 1657-1662 (1994)) coupled with ligase-mediated PCR (LMPCR) has permitted detection of certain human *p53* mutations at a sensitivity of about 1%. The most complicated and least general methods, such as RFLP-PCR, need to be employed whenever the mutation is present in a small fraction of the templates (<1%). In addition, only RFLP/PCR in its pure form amplifies internal target sequences, permitting subsequent verification of the mutation by sequencing. Mismatch-specific TaqMan PCR, an embodiment of the present invention, also produces a product containing the mutant allele DNA which can be verified by sequencing.

The present invention relates to methods for enhancing allele-specificity, especially for transition and small

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frameshift mutations. The present invention more specifically relates to inclusion of a thermostable mismatch binding protein and a thermostable protein which enhances specific binding of the thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid in a PCR amplification procedure. Examples of thermostable mismatch binding proteins include Apy, Tma, Tth and Taq MutS proteins. Examples of thermostable proteins which enhance specific binding of the thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid include Apy, Tma and Tth MutL proteins. A simple assay would be more amenable to automation using highly-parallel "classical" or chip-based amplification technologies. Chip-based technologies can be used to provide an array of blocking oligonucleotides, permitting multiplex mismatch-specific TaqMan PCR.

In one embodiment, the invention relates to a method for enhancing mismatch-specific TaqMan PCR. As used herein, "TaqMan PCR" refers to a PCR assay based on the "Taqman" system described by Holland, P.M. et al., *Proc. Natl. Acad. Sci. U.S.A.* 88: 7276-7280 (1991). In a particular embodiment, Apy MutS or Tma MutS binds specifically to a heteroduplex internal oligonucleotide-template complex containing a GT transition mismatch or a small bulge loop and not to a perfectly matched internal oligonucleotide-template complex, thus interfering with propagation of polymerization (e.g., blocking DNA polymerization) from the mismatched template during each PCR cycle. Addition of Apy MutL or Tma MutL enhances mismatch-specific TaqMan PCR. For example, addition of Apy MutL or Tma MutL enhances binding of Apy MutS and Tma MutS to bulge loops in the heteroduplex internal oligonucleotide-template nucleic acid. Alternatively, addition of Apy MutL or Tma MutL stabilizes complexes produced by binding of Apy MutS or Tma MutS to a bulge loop

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in a heteroduplex nucleic acid. For detectable types of mutations, mismatch-specific TaqMan PCR is amenable to automation using highly-parallel "classical" or chip-based amplification technologies. Chip-based technologies can be used to provide an array of blocking oligonucleotides, permitting multiplex mismatch-specific TaqMan PCR.

For every AC mismatch on one nucleic acid strand, there is a GT mismatch on the other nucleic acid strand. In fact, a specific GT mismatch can always be formed between a TaqMan oligonucleotide of one polarity and a wild-type sequence, even in the case of transversion mutations. The specificity will then depend upon the extent to which the mutant allele could be amplified with a mismatched primer containing a mismatch other than GT.

Allele-specific oligonucleotides forming a GT mismatch can be synthesized, although thermostable mismatch binding proteins can bind to other types of heteroduplexes, which binding is enhanced in the presence of one or more thermostable proteins that enhance binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid.

In another embodiment, the invention relates to a method for enhancing primer-directed allele-specific PCR. In a particular embodiment, Apy MutS or Tma MutS binds specifically to a heteroduplex primer-template complex containing a GT transition mismatch (for every AC mismatch there is a GT mismatch) or a small bulge loop and not to a perfectly matched primer-template complex, thus interfering with initiation of polymerization from the mismatched template. Addition of Apy MutL or Tma MutL enhances primer-directed allele-specific PCR. For example, addition of Apy MutL or Tma MutL enhances binding of Apy MutS and Tma MutS to bulge loops in the heteroduplex primer-template nucleic acid.

Allele-specific primers forming a GT mismatch can be synthesized, although thermostable mismatch binding proteins, can bind to other types of heteroduplexes, which binding is enhanced in the presence of one or more
5 thermostable proteins that enhance binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid. Of greater importance, any selection against primer-template mismatches throughout the length of a primer-template complex should translate into fewer
10 improper extension products for all PCR reactions. Compatibility between allele-specific amplification conditions and long PCR conditions (Cheng, S. et al., *Proc. Natl. Acad. Sci. USA* 91: 5695-5699 (1994)) is considered.

Isolated, recombinant thermostable MutL protein or a
15 portion thereof, and suitable fusion proteins can be used in methods for enhancing allele-specificity (e.g., in methods for enhancing mismatch-specific TaqMan PCR, such as in methods for detecting mismatches formed between heteroduplex template-oligonucleotide nucleic acids, and in
20 methods for enhancing primer-directed allele-specific PCR).

The present invention also relates to methods for selecting against amplification of mismatches between complementary strands. Specifically, the present invention relates to methods for selecting against amplification of
25 heteroduplex nucleic acid.

Fidelity of DNA Replication

The present invention further relates to methods of reducing DNA misincorporation (i.e., improving fidelity of DNA replication) in an amplification reaction.

30 Replication errors are frequent with all thermostable polymerases, even using the optimum conditions (Eckert, K.A. and Kunkel, T.A., *PCR. Methods. Appl.* 1: 17-24 (1991); Ling, L.L. et al., *PCR. Methods. Appl.* 1: 63-69 (1991)). Comparing optimal conditions, the 3'→5' editing exonuclease

activity of a polymerase will decrease PCR errors by no more than 2-5 fold. The majority of errors introduced during PCR amplification are transitions (Keohavong, P. et al., *PCR. Methods. Appl.* 2: 222-292 (1993)). Improvement of fidelity depends upon the ability of MutS to bind heteroduplex nucleic acid tightly and provide a nucleus for renaturation following the strand-separation step of PCR. MutL can enhance MutS binding to heteroduplex nucleic acid. A renatured PCR product would not act as a template for subsequent amplification. Apy and Tma MutS and MutL proteins are ideal candidates for use in PCR because they were cloned from hyperthermophiles.

The ultimate specificity of mismatch-specific TaqMan PCR can be determined by the frequency at which wild-type templates are amplified, in spite of the selection against them, and at which misincorporation produces the mutant sequence.

Misincorporation

Fidelity with and without Apy or Tma MutS and MutL can be assayed by determining the frequency of mutations introduced during amplification of *lacI^q* which prevent expression of a functional lac repressor protein.

As described in the Examples, a simple blue-white screen was developed for measuring PCR fidelity. A plasmid derived from pUC19 was kindly provided by Dr. Y. Ioannou (Mount Sinai School of Medicine) in which the 880 bp sequence from the AatII site (GACGTC ...) to the AflIII site (... ACATGT) was replaced by GACTCTAGAGGATCCATGT (SEQ ID NO:16), introducing an XbaI site and a BamHI site. pET11a (Novagen, Inc.) was cleaved with BstYI to produce ends compatible with BamHI and ligated into the BamHI-cleaved modified pUC19 vector. A clone was selected which contained the pET11a fragment from 748 to 1961, containing the complete *lacI^q* gene, and was designated pUC17I. E.

coli KL318 (K.B. Low) was obtained from the *E. coli* Genetic Stock Center (#4350). This *lacI*22 strain was constitutive for expression of *lacZ* and able to cleave 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) to produce a blue color.

5 Transformation by pUC17I led to expression of *lacI*^q and repression of *lacZ*. One set of PCR primers, 5' AUGAUGAUGAUGAUCGCACATTTCCTCCGAAAAGTG 3' (SEQ ID NO:17) and 5' AUCAUCAUCAUCAUGCGCGGAACCCCTATTTGT 5' (SEQ ID NO:18), was used to amplify pUC17I. The products were

10 phenol/chloroform extracted and purified on Millipore Ultrafree MC 30,000 NMWL filters before digestion with one unit uracil-DNA glycosylase (UDG) in 30 mM Tris (pH 8.3), 50 mM KCl, 5 mM MgCl₂ for 1 hr at 37°C. The circularized products were introduced into *E. coli* KL318 by

15 electroporation. An alternative set of PCR primers was prepared which required restriction endonuclease cleavage and ligation before electroporation. In both cases, the cells were propagated at several dilutions on plates containing ampicillin, isopropyl- β -D-thiogalactopyranoside

20 (IPTG) and X-gal. In both cases, the presence of a subset of blue colonies indicated failure to produce active *LacI*^q due to a mutation introduced during PCR. There was little advantage of one set of primers and cloning conditions over the other.

25 Amplification reactions can be carried out with or without added Apy or Tma MutS + MutL protein. The relative numbers of blue colonies is a measure of the efficacy of the thermostable MutS + MutL proteins in blocking mismatch-containing PCR products, resulting from polymerization

30 errors, from acting as templates in subsequent rounds of PCR.

Several thermostable DNA polymerases (e.g., Taq, Vent) may be suitable in the amplification reaction. Initially, published PCR conditions known to optimize for fidelity of

35 a particular polymerase can be used, and PCR conditions can

be varied to verify optimum polymerase fidelity. Subsequently, each of the appropriate variables affecting PCR can be modified to optimize for replication fidelity in the presence of Apy and Tma MutS \pm MutL, even if polymerase fidelity in the absence of a thermostable MutS \pm MutL protein is suboptimal. The optimized results in the presence of thermostable MutS \pm MutL proteins can be compared to the optimized results without MutS \pm MutL to determine the fold improvement in PCR fidelity for the two MutS and MutL proteins for each of the polymerases.

Decreased Stuttering/Slippage At Dinucleotide and Trinucleotide Repeats

Fidelity with and without Apy or Tma MutS and MutL can also be assayed by determining the extent of frameshift mutation ("stuttering"/"slippage") during amplification of di- and trinucleotide repeats. In the absence of these repeats, most of the replication errors are known to be transitions. For di- and trinucleotide repeats, most of the errors are known to be frameshifts.

Amplification of the highly polymorphic dinucleotide and trinucleotide repeats in human genomic for gene mapping usually results in ladders of bands thought to be due to polymerase "stuttering"/"slippage." D10S183 (MFD200, 124-158 bp) and D4S171 (MFD22, 143-161 bp) were used to amplify human genomic DNA. One primer was labeled with ^{32}P . The products were separated on DNA sequencing gels and analyzed by autoradiography. The expected ladders of bands were observed. It is reasonable to expect that one or more sets of primers for highly polymorphic trinucleotide repeats can also be found which will give reproducible ladders with a spacing of 3 nucleotides.

Whatever the mechanism of stuttering/slippage, the ladders must reflect denaturation and amplification of PCR intermediates with 2 or 3 nucleotide loops similar to those

found in heteroduplexes formed between pUC19Δ3 and pUC19Δ1 or pUC19GC, respectively. In preliminary experiments, MutS alone was ineffective at reducing stuttering. However, if thermostable MutS + MutL proteins prevents extension of slipped templates, these ladders can be reduced or eliminated, thus making the use of these polymorphic markers more convenient for genomic mapping and fingerprinting.

Amplification of representative di- and trinucleotide repeat regions of human DNA can be carried out in the presence and absence of Apy or Tma MutS + MutL to optimize conditions. Each of the appropriate variables affecting PCR can be modified to optimize for replication fidelity in the presence of Apy and Tma MutS + MutL, as measured by reduction or elimination of stuttering/slippage.

Heteroduplex Binding and Detection

Many of the DNA manipulations described herein involve standard techniques and procedures (Sambrook, J. et al., *Molecular Cloning, A Laboratory Manual*, 2nd edition, Cold Spring Harbor University Press, New York, 1989).

As described herein, the mismatch binding assay (also referred to herein as the gel shift binding assay or the gel shift assay) was used to evaluate the MutL proteins of the present invention for the ability to enhance binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid. Proteins other than MutL can also be evaluated for the ability to enhance binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid using this assay. The mismatch binding assay is also used to evaluate thermostable mismatch binding proteins for specific binding to bulge loops in a heteroduplex nucleic acid. Protein complexes can also be evaluated for specific binding to bulge loops in a heteroduplex nucleic acid using the gel shift assay.

As used herein, a "protein complex" includes a molecular complex of two or more proteins.

As described in the Examples, to make heteroduplex substrates for use in evaluating thermostable MutS and MutL proteins for specific binding to bulge loops in a heteroduplex nucleic acid, several modifications were introduced into pUC19 by replacing the *Kpn*I to *Pst*I segment of the polylinker. In pUC19GC, the *Bam*HI site GGATCC in the sequence GGGGATCCTC (SEQ ID NO:10) was modified to substitute a C for the first T to yield GGGACCCTC (SEQ ID NO:11). The resultant plasmid gained an *Ava*II site. In pUC19A1, a T was inserted into the pUC19GC polylinker sequence GGGACCCTC to yield GGGGATCCCCTC (SEQ ID NO:12) and reconstitute the *Bam*HI site. In pUC19A3, a T and two Cs were inserted into the pUC19GC polylinker sequence GGGACCCTC to yield GGGGATCCCCCTC (SEQ ID NO:13) and again reconstitute the *Bam*HI site. The sequences were verified.

In addition to pUC19GC, pUC19CG and pUC19TA can be similarly constructed to study transversion substitutions using the same oligonucleotide probes.

PCR products of 337-340 bp were synthesized from pUC19, pUC19GC, pUC19A1 and pUC19A3 using 5' TACGCCAGCTGGCGAAAGGG 3' (SEQ ID NO:14) and 5' AATGCAGCTGGCAGCAGG 3' (SEQ ID NO:15), where the *Pvu*II sites are underlined. PCR products up to 2.7 kb can be prepared using appropriate primers. For some experiments, one of the primers was labeled with ³²P using T4 polynucleotide kinase to allow quantitation of products.

PCR products of 337-340 bp can be synthesized from pUC19CG and pUC19TA using 5' TACGCCAGCTGGCGAAAGGG 3' (SEQ ID NO:14) and 5' AATGCAGCTGGCAGCAGG 3' (SEQ ID NO:15), where the *Pvu*II sites are underlined. PCR products up to 2.7 kb can be prepared using appropriate primers.

Heteroduplexes were formed in PCR and similar buffers from various ratios of two different PCR products by

denaturation at about 97°C and annealing at about 67°C (Wetmur, J.G., *Crit. Rev. Biochem. Mol. Biol.* 26: 227-259 (1991)). Heteroduplexes between pUC19GC (or pUC19) and pUC19Δ3 were easily separated from homoduplexes on a 6% polyacrylamide gel. Heteroduplexes between pUC19Δ1 and pUC19Δ3, while less separated from homoduplexes because of a loop size of two rather than three, were easily distinguished. Heteroduplexes between pUC19GC (or pUC19) and pUC19Δ1, as well as heteroduplexes between pUC19 and pUC19GC, could not be distinguished from homoduplexes using this gel system. In particular, the homoduplexes, differing by only 3 base pairs, had almost identical mobilities. The heteroduplexes had reduced mobility. Denaturation and fast cooling prevented complete renaturation and revealed a slower-moving denatured DNA band. Addition of Apy MutS protein led to a gel shift of the heteroduplex band and appearance of a new band for the complex. Denaturation and fast cooling in the presence of the thermostable Apy MutS demonstrated that the specific binding to the heteroduplex was preserved.

Heteroduplexes were formed between pUC19GC prepared with one labeled primer and unlabelled pUC19Δ1 or pUC19 using the unlabeled molecule in excess so that most of the label is in heteroduplex and not homoduplex. Similarly, heteroduplexes can be formed between pUC19GC prepared with one labeled primer and unlabelled pUC19CG or pUC19TA using the unlabeled molecule in excess so that most of the label is in heteroduplex and not homoduplex. *Ava*II cleavage was tested for the ability to deplete residual homoduplexes without affecting the heteroduplexes.

Heteroduplexes can also be formed by reversing the choice of labeled PCR product and renaturation driver. For example, heteroduplexes can be formed by using labeled pUC19. *Bam*HI cleavage can similarly be tested for the ability to deplete residual homoduplexes without affecting

the heteroduplexes. Labeled heteroduplexes were also formed using pUC19GC and pUC19Δ3.

Heteroduplex formation with duplex molecules leads to two types of mismatches. For example, with pUC19 plus
5 pUC19GC heteroduplexes, GT and AC mismatches were created simultaneously. Hybridization of the plus strand of pUC19GC with the complementary strand of pUC19 DNA leads to an AC mismatch, whereas hybridization of the plus strand of pUC19 with the complementary strand of pUC19GC DNA leads to
10 a GT mismatch. Heteroduplex formation between pUC19Δ1 and pUC19GC leads to molecules with unpaired A or T residues. Heteroduplex formation between pUC19Δ3 and pUC19GC leads to molecules with three unpaired GGA or TCC residues. These mismatches were evaluated independently by the choice of
15 radiolabeled primer, using the gel shift assay.

MutS binding assays employed a 1:20 dilution of each of the heteroduplex mixtures or homoduplex controls from PCR buffer into 20 mM Tris, pH 7.5, 5 mM MgCl₂, 0.1 mM DTT, 0.01 mM EDTA to give approximately 5 μg/ml total DNA.
20 Thermostable MutS and MutL proteins purified to homogeneity were used in the assays. However, using the MutS binding assays described, any protein purified to homogeneity can be evaluated for specific binding to bulge loops in a heteroduplex nucleic acid. In addition, using the MutS
25 binding assays described, any protein purified to homogeneity can be evaluated for the ability to enhance specific binding of a second protein or combination of proteins to bulge loops in a heteroduplex nucleic acid.

Variables in the MutS binding assays include protein
30 concentration (stoichiometry), temperature, pH, added KCl and added Mg⁺⁺. After incubation in the presence or absence of thermostable mismatch repair proteins (MutS + MutL), the products were separated by electrophoresis at 25 V/cm for 30 minute on a 6% polyacrylamide gel at 4°C in
35 0.2 x TBE and analyzed either by ethidium bromide staining

and UV fluorography or by autoradiography. As used herein, "thermostable mismatch repair proteins" refer to thermostable proteins that are associated with nucleic acid mismatch repair and include thermostable mismatch binding proteins (e.g., thermostable MutS proteins), thermostable proteins that enhance binding of a thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid (e.g. thermostable MutL proteins), and thermostable proteins associated with nucleic acid strand discrimination (e.g., thermostable MutH proteins).

The effects of temperature, pH, and salts in the loading and running buffers of the gel shift assay can be adjusted to provide for a set of standard assay conditions where specific binding to bulge loops of the thermostable mismatch repair proteins to be evaluated is not affected by the assay conditions. For the assay to have no effect, protein exchange must not take place during the assay. To determine the assay conditions most permissive of sample variability, identical measurements can be carried out with and without unlabeled mismatch-free DNA and/or heteroduplexes added to the loading buffer. In some measurements, the unlabeled DNA can be added to the incubation mixture before preparation for electrophoresis.

In preliminary experiments where electrophoresis was carried out at 4°C, which may not be desirable with thermophilic proteins, addition of mismatch-free duplex DNA was necessary to suppress non-specific binding of Apy MutS to homoduplex DNA.

To investigate thermostability of Apy MutS and MutL, Tma MutS and MutL proteins, and other thermostable MutS and Mut L proteins, after incubation at constant temperature in PCR buffer, aliquots of the MutS and Mut L proteins were removed as a function of time and tested for binding activity in the standard assay.

One variable in the specificity of MutS + MutL binding is MutS and MutL stoichiometry to heteroduplex DNA. Thus, to investigate specificity of MutS + MutL binding to the set of heteroduplexes, addition of competing mismatch-free superhelical or linear dsDNA, or ssDNA, were used as an assay for non-specific binding. The linear dsDNA can be varied in size to test for end effects. Other variables include incubation temperature and time, pH, KCl and Mg⁺⁺ concentrations.

10 MutS proteins all contain a Walker motif, GxxxxGKS, which has been implicated in NTP binding. Although inclusion of ATP or ATPγS in the Apy MutS binding assay to a 3 nucleotide loop had no effect on the binding stoichiometry, possible effects on affinity for other
15 mismatches, such as those resulting from transversions, can be determined.

To investigate thermostability of each of the complexes formed between Apy MutS + MutL and Tma MutS + MutL with the set of radiolabeled heteroduplex nucleic acids, after complex formation, unlabeled PCR product identical to the labeled PCR product used for heteroduplex nucleic acid formation can be added to restore 1:1 stoichiometry. After incubation at a particular temperature, renaturation to completion and
25 deproteinization, the fraction of newly-formed unlabeled heteroduplex nucleic acid, up to 50% of the total DNA, will reflect homoduplex nucleic acid strand separation and the fraction of newly-formed labeled homoduplex nucleic acid, up to 50% of the labeled DNA, will reflect mismatch binding
30 protein-heteroduplex nucleic acid complex strand separation. The relative strand-separation temperatures of heteroduplex nucleic acid complexes and uncomplexed homoduplex nucleic acids in conditions compatible with PCR can thus be determined.

Kinetics of Heteroduplex Binding

The reverse rate (dissociation rate) can be determined by measuring the rate of exchange from a MutS ± MutL complexed with a radiolabeled heteroduplex nucleic acid to a competing unlabeled heteroduplex nucleic acid using a variety of solvent conditions. For example, in preliminary experiments, 1 mM ATPγS was observed to retard dissociation exchange of Apy MutS from a pUC19-pUC19Δ3 heteroduplex DNA to competing DNA. The pUC19-pUC19Δ3 heteroduplexes with only MutS bound are sufficiently stable to permit gel-shift analysis and can be used as the unlabeled heteroduplex nucleic acid for investigating the complete set of radiolabeled heteroduplex nucleic acids. To determine whether exchange requires dissociation of mismatch binding proteins from the labeled heteroduplex DNA before binding to competing DNA, the effects of the concentrations of specific competing heteroduplex DNA or non-specific competing native DNA were determined. Thus, the optimum conditions favoring heteroduplex nucleic acid stability consistent with specificity and PCR can be found.

The forward rate (binding rate) can be determined using a variety of solvent conditions where the dissociation rate is slow. Binding can be terminated as a function of time by adding competing DNA, and the fraction of labeled heteroduplex DNA complexed to mismatch binding proteins can be determined. The forward rate constant for MutS ± MutL binding to a mismatch cannot be greater than approximately $2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, the diffusion control limit, unless binding is mediated through exchange from non-specific binding sites. For example, the half-time for the diffusion controlled reaction would be approximately 0.6 sec at 12.5 nM target each of heteroduplex DNA (e.g. 50% of 100 ng/20 μl) and MutS (50 ng/20 μl). Lower concentrations permit determination of binding rate constants. Thus, the MutS ± MutL concentration(s) necessary for specific, stable

and rapid mismatch binding in conditions compatible with PCR can be found. To be effective, this binding to a mismatch must occur before the DNA polymerase initiates DNA polymerization in primer-directed allele-specific PCR
5 primers or copies the template in mismatch-specific TaqMan PCR.

Nuclease Protection Assays

Footprints of Apy and Tma MutS + MutL binding to the set of radiolabeled heteroduplex nucleic acids can be
10 determined by electrophoresis on sequencing gels following limited endonuclease digestion of heteroduplex nucleic acids labeled first at one end and then at the other. Footprinting can also be attempted using the 5'→3' exonuclease activity of thermostable Taq DNA polymerase, in
15 the absence of dNTPs, and the 3'→5' exonuclease activity of thermostable Vent DNA polymerase in a manner akin to the use of the 3'→5' exonuclease activity of T7 DNA polymerase with *E. coli* MutS (Ellis, L.A. et al., *Nucleic Acids Res.* 22: 2710-2711 (1994)). Thus, the footprints can be
20 obtained for both mismatch and bulge-loop defects. These footprints aid in the design of TaqMan oligonucleotides and allele-specific PCR primers.

Other Mismatches

Transitions and small frameshifts are the mutations
25 known to be the most effective mismatch binding protein substrates. However, transversion mutations can be effective mismatch binding protein substrates. Optimal conditions for binding of mismatch binding proteins to TC, CC, TT, GA, GG and AA mismatches can be tested after the
30 design and production of additional PCR templates.

Primer Extension Assays

Mismatched TaqMan primers (mismatches or bulge loops) can be used to form complexes with Apy MutS plus MutL as well as Tma MutS plus MutL. Radiolabeled primer extension products synthesized by Taq or Tth polymerase and its derivatives (e.g. Stoffel fragment and other enzymes lacking 5' -> 3' exonuclease activity) and blocked by these complexes can be analyzed by electrophoresis on sequencing gels. In designing TaqMan oligonucleotides, to determine the closest distance of approach of the polymerase to the mismatch, a set of TaqMan oligonucleotides can be constructed with increasing 5' extensions well beyond the mismatch position.

Mismatch-Specific TaqMan PCR

Allele-specific amplification with a mismatched internal oligonucleotide demonstrates that propagation of polymerization can be inhibited by forming a mismatch binding protein-internal duplex mismatch complex. To optimize the choice of DNA polymerases, thermostable mismatch binding proteins and internal oligonucleotide design in terms of both PCR sensitivity and allele specificity, DNA polymerization through matched and mismatched TaqMan primer-template complexes may be examined. Unlike the primer-directed allele-specific system, MutS- plus MutL-mediated selective amplification occurs at each PCR cycle. The assay (TaqMan PCR) is based on the "TaqMan" system first described by Holland, P.M. et al., *Proc. Natl. Acad. Sci. U.S.A.* 88: 7276-7280 (1991). As used herein, the terms "TaqMan oligo", "TaqMan oligonucleotide" and "TaqMan primer" refer to an internal oligonucleotide. As used herein, an "internal oligonucleotide" is an example of a blocking oligonucleotide.

In one set of experiments, the PCR template mixture is a serial dilution of pUC19GC with constant concentrations of pUC19, pUC19A1 or pUC19A3. Commercial human DNA is added to 1 μ g/reaction. One set of PCR primers can be the two PvuII-containing primers described previously (SEQ ID NO:14 and SEQ ID NO:15). Additional primers can be synthesized to produce longer PCR products. A third TaqMan oligonucleotide can match the AvaII-containing region of pUC19GC or the corresponding region of one of the other templates.

Results with Taq DNA polymerase amplification of pUC19GC and pUC19A3 in the presence of a TaqMan oligonucleotide, Apy MutS and Apy MutL are presented in Example 8 (see the Table) and demonstrate that the complete TaqMan system works.

In other experiments, pUC19 is subjected to serial dilution. The TaqMan oligonucleotide can match the BamHI containing region of one of the templates. Templates that can be held at constant concentration are described above and include pUC19GC, pUC19A1, pUC19A3, pUC19CG and pUC19TA.

Many TaqMan oligonucleotides can be synthesized and tested, with the design informed by the experiments described herein. These oligonucleotides can contain a 3' terminal phosphate residue to prevent extension by Taq DNA polymerase or its derivatives, which lack 3'→5' exonuclease activity.

When present at a concentration in excess of the PCR primer concentrations, TaqMan oligonucleotide-template complexes form efficiently, and bound TaqMan oligonucleotide is degraded by the 5'→3' exonuclease activity of Taq polymerase during the polymerization step of PCR. In the case of derivatives like Stoffel fragment that lack 5'→3' exonuclease activity, the TaqMan oligonucleotide is displaced. All of the assay conditions can be tested for efficient degradation or displacement of

radiolabeled Taqman oligonucleotides. Because only the PCR products from the pUC19GC template can be cleaved by *Ava*II and only the PCR products from pUC19, pUC19Δ1 or pUC19Δ3 can be cleaved by *Bam*HI, the relative yields of the two PCR products can be determined by cleavage with *Ava*II, *Bam*HI or both enzymes, gel electrophoresis, and fluorography or autoradiography.

Apy and Tma MutS and MutL proteins can be examined independently for their ability to recognize TaqMan oligonucleotide-template complexes and inhibit the propagation step of polymerization during PCR. Other proteins can also be examined for their ability to recognize TaqMan oligonucleotide-template complexes and inhibit the propagation step of polymerization during PCR or for their ability to enhance binding of thermostable mismatch binding proteins to TaqMan oligonucleotide-template complexes and thus enhance blocking of the propagation step of polymerization during PCR.

Taq DNA polymerase has a processivity of about 60 nucleotides at the maximum rate of polymerization (about 50 nucleotides/second). When Taq polymerase encounters a mismatch binding protein-heteroduplex nucleic acid complex, the most likely scenario is dissociation of the polymerase. However, if a bound polymerase is capable of displacing the mismatch binding protein-heteroduplex nucleic acid complex, altering variables such as (i) the dilution of the mismatched template in the carrier DNA (the complexity), (ii) the nature of the mismatch and bulge loops formed between the Taqman oligonucleotides and the template (e.g., pUC19 or pUC19GC), (iii) the detailed position of the mismatch in the TaqMan oligonucleotide, (iv) the spacing between the initiation PCR primer and the TaqMan oligonucleotide, (v) the DNA polymerase, (vi) the MutS + MutL source, (vii) the number of PCR cycles, (viii) the cycling conditions, (ix) salt and dNTP concentrations, and

(x) the absolute and relative concentrations of the DNA polymerase, the MutS, the MutL and the TaqMan oligonucleotide, in a manner leading to reduced processivity, should lead to dissociation. Thus, these variables can be optimized in the mismatch-specific TaqMan PCR system.

The TaqMan reader manufactured by the Applied Biosystems Division of Perkin-Elmer can be used to investigate high throughput screening methods. This reader detects fluorescent products in a 96-well plate after transfer from PCR tubes in a compatible format. One possible format for its use in testing the variables described above with Taq polymerase and derivatives retaining the 5'→3' exonuclease activity is to use a second TaqMan oligonucleotide, containing a fluor and quencher, which precisely matched a new sequence cloned into pUC19 and pUC19GC. This format allows use of a single fluor-quencher TaqMan oligonucleotide for all of the experiments.

In addition to specificity, it is important to achieve the highest possible sensitivity. One approach to achieving single molecule sensitivity is preamplification for several or many cycles before the addition of the TaqMan primer, MutS and MutL. Preamplification might be necessary if MutS + MutL inhibits PCR of matched templates at all and/or if more than one mutation were to be detected in a single amplicon. Mismatch-specific TaqMan PCR technology is amenable to automation. On a chip, screening for many mutant alleles can easily be accomplished in parallel, and preamplified DNA is the obvious input. However, this design may be limited if PCR misincorporation errors lead to false positive results. Thus, preamplified products from a single template and mismatched primers differing by a single transition can be tested as input. The products that escape selection can be tested for the appearance of a restriction endonuclease cleavage site.

Because the TaqMan oligonucleotide is not incorporated into the amplification product, the same selection takes place at each cycle, permitting geometric selection. In addition to selection at each PCR cycle, another advantage of inhibition of propagation rather than initiation is that more time will be available for the formation of the thermostable MutS-heteroduplex nucleic acid complex before the critical polymerase inhibition step takes place. This simple closed tube technology for detecting mutant alleles in a vast excess of normal alleles has important applications in the study of cancer and cancer epidemiology.

Primer-Directed Allele-Specific Amplification

Allele-specific amplification with matched primers demonstrates that binding of a thermostable mismatch binding protein to a variety of mismatched primer-template complexes inhibits initiation of polymerization.

In one embodiment of primer-directed allele-specific amplification, the PCR template is a mixture containing one of the pUC19 derivatives described previously (especially pUC19GC and pUC19Δ1) and pMS19, a derivative of pUC19 with inserts of 35 bp at both the *EcoRI* and *HindIII* sites but with a polylinker region identical to pUC19 (Weinstock, P.H. and Wetmur, J.G., *Nucleic Acids Res.* 18: 4207-4213 (1990)). One primer was selected from the *PvuII*-containing primers described herein (SEQ ID NO:14 or SEQ ID NO:15). The reverse primer was synthesized to match either the *BamHI*-containing region of pMS19 or the corresponding region of one of the pUC19 derivatives. Two types of primer-template mismatches can thus be prepared and each seen in two contexts. The additional 35 bp in PCR products derived from pMS19 permitted easy identification of products following polyacrylamide gel electrophoresis and ethidium bromide staining. Quantitative autoradiography

can also be employed to identify products. In addition to mismatch type (especially GT and AC mismatches and single frameshift mutations), efficiency of inhibition of amplification by MutS + MutL binding also depends on PCR
5 conditions and the location of the mismatch within the primer.

Mismatches not only affect the melting temperature of the primer-template complex (Wetmur, J.G., Crit. Rev. Biochem. Mol. Biol. 26:227-259 (1991)), but also the
10 initiation of extension by the thermostable DNA polymerase. For each assay, template ratios may need adjustment to produce equal yields of the PCR products from the two templates in the absence of Apy or Tma MutS + MutL. Using this system, a 10-20 fold improvement was achieved in
15 allele-specific PCR with mismatches 7-9 nucleotides away from the 3' end of the primer. Typically, mismatches that far from the polymerase binding site have little effect on initiation efficiency.

The effect of Apy and Tma MutS + MutL on the ratio of
20 PCR products can be examined as a function of MutS + MutL concentration and thermostable DNA polymerase concentration. This ratio must be high enough to permit nearly complete MutS + MutL binding to first-round primer template complexes before the polymerase has an opportunity
25 to bind and initiate extension. Cycling parameters can be adjusted as appropriate. Input template concentration and KCl and Mg⁺⁺ concentrations can also be adjusted. Compatibility of the system with dI and dU incorporation may also be examined.

30 As used herein, the terms "template", "template nucleic acid", "target template" and "target nucleic acid" are defined as a nucleic acid, in purified or nonpurified form, which comprises the specific sequence desired (nucleotide sequence of interest). Any nucleic acid can be
35 utilized as the template. The nucleic acid can be obtained

from any source, for example, from plasmids such as pBR322, from cloned DNA or RNA, or from natural DNA or RNA from any source, including bacteria, yeast, viruses, organelles, and higher organisms such as plants and animals. DNA or RNA may be extracted from blood, tissue material such as chorionic villi, or amniotic cells by a variety of techniques. (See, e.g., Sambrook, J. et al., *Molecular Cloning, A Laboratory Manual*, 2nd edition, Cold Spring Harbor University Press, New York, 1989). Thus, the template may be DNA or RNA, including messenger RNA, which DNA or RNA may be single-stranded or double-stranded. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture can also be used, as can nucleic acids produced from a previous amplification reaction (using the same or different primers). The template may be only a fraction of a large molecule or can be present initially as a discrete molecule, so that the specific sequences constitutes the entire nucleic acid.

If the nucleic acid is double-stranded, it is necessary to separate the strands of the nucleic acid before it can be used as the template. This strand separation can be accomplished by any suitable denaturing method including physical, chemical or enzymatic means. One preferred physical method of separating the strands of the nucleic acid involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation involves temperatures ranging from about 90 to 105°C for times generally ranging from about 0.5 to 5 minutes. Preferably the effective denaturing temperature is 90-100°C for 0.5 to 3 minutes. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or the enzyme RecA, which has helicase activity and in the presence of riboATP is known to denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases are described by Kuhn

Hoffmann-Berling, *CSH-Quantitative Biology*, 43: 63 (1978), and techniques for using RecA are reviewed in C. Radding, *Ann. Rev. Genetics*. 16: 405-437 (1982). The denaturation produces two separated complementary strands of equal or
5 unequal length.

The term "oligonucleotide" as used herein is defined as a molecule comprised of 8 or more deoxyribonucleotides and typically 20-40 deoxyribonucleotides. Its exact size will depend on many factors, which in turn depend on the
10 ultimate function or use of the oligonucleotide. The oligonucleotide may be derived synthetically or may be isolated from natural sources by cloning, for example.

As used herein, an oligonucleotide which is designed to be completely complementary to a specific nucleotide
15 sequence of interest hybridizes to the complementary region of the strand of the template which includes the nucleotide sequence of interest to form a homoduplex nucleic acid. The oligonucleotide which is designed to be completely complementary to a specific nucleotide sequence of interest
20 hybridizes to a strand of a nucleic acid which does not include the nucleotide sequence of interest to form a heteroduplex nucleic acid. An oligonucleotide which is designed to be completely complementary to a specific nucleotide sequence of interest can be a primer, a blocking
25 oligonucleotide or a probe.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest for example, or produced synthetically, which is capable of acting as a point of
30 initiation of synthesis when placed under conditions in which primer extension is initiated. Synthesis of a primer extension product which is complementary to a nucleic acid strand is usually initiated in the presence of four different nucleoside triphosphates and an inducing agent
35 such as DNA polymerase in an appropriate buffer and at a

suitable temperature and pH. The specific buffer, temperature and pH depend on the inducing agent and the amplification method used.

The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer, as used in nucleic acid amplification reactions, is single-stranded.

10 Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and use of

15 the method. For example, for diagnostics applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides. For other applications, the oligonucleotide primer is

20 typically shorter, e.g., 8-15 nucleotides. Such short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with template.

The term "blocking oligonucleotide" refers to an oligonucleotide, whether occurring naturally as in a

25 purified restriction digest or produced synthetically, which is capable of inhibiting propagation of polymerization of a primer extension product (i.e., inhibiting elongation of the extension product) when placed under conditions in which primer extension product is

30 elongated. Propagation of a primer extension product which is complementary to a nucleic acid strand typically occurs in the presence of four different nucleoside triphosphates and an inducing agent such as DNA polymerase and at a suitable temperature and pH.

The blocking oligonucleotide is preferably single stranded for maximum efficiency in amplification, but may alternatively be partially complementary. For DNA amplification methods, the blocking oligonucleotide is an
5 oligodeoxyribonucleotide. The blocking oligonucleotide must be sufficiently long to permit formation of the heteroduplex template-blocking oligonucleotide complex. The exact lengths of the blocking oligonucleotides will depend on many factors, including temperature, source of
10 primer and use of the method. The blocking oligonucleotide must be modified at the 3' end to prevent its function as a primer (e.g., modified with 3' phosphate with Taq polymerase which lacks 3'->5' editing exonuclease activity). The "Taqman oligonucleotide" or "internal
15 oligonucleotide" is an example of a blocking oligonucleotide.

The term "probe" as used herein includes an oligonucleotide, whether occurring naturally as in a purified restriction digest for example, or produced
20 synthetically, which is capable of being covalently fused or ligated together into a product which is complementary to a nucleic acid strand of the target template when placed under conditions in which product formation is initiated. Formation of a product which is complementary to a nucleic
25 acid strand is initiated in the presence of a fusing agent such as DNA ligase in an appropriate buffer and at a suitable temperature and pH. The specific buffer, temperature and pH will depend on the fusing agent and the amplification method used.

30 The probe is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the probe is first treated to separate its strands before being used to prepare amplified products. The probe, as used in nucleic
35 acid amplification reactions, is single-stranded.

Preferably, the probe is an oligodeoxyribonucleotide. The probe must be sufficiently long to provide the desired specificity (i.e., to avoid being hybridized to random sequences in a sample). Typically, probes on the order of 5 15 to 100 bases serve this purpose. The exact lengths of the probes will depend on many factors, including temperature, source of primer and use of the method.

In one embodiment, oligonucleotides designed to be completely complementary to a specific nucleotide sequence of interest, whether a primer, blocking oligonucleotide, or probe, can be designed for use in pairs, one oligonucleotide to anneal to and block the amplification of each complementary strand of the template, for example, in a control sample (i.e., in a sample of nucleic acids known 15 to not include the nucleotide sequence of interest). Complementary overlap between oligonucleotides designed to be completely complementary to a specific nucleotide sequence of interest should be minimized to avoid the stable annealing of the oligonucleotides to each other.

20 In another embodiment, oligonucleotides designed to be completely complementary to a specific sequence of interest, whether a primer, blocking oligonucleotide, or probe, can be designed for use as a single oligonucleotide, annealing to and blocking the amplification of one strand 25 of the template, for example, in a control sample (i.e., in a sample of nucleic acids known to not include the nucleotide sequence of interest).

The following is an illustration of the use of MutS and MutL proteins with oligonucleotides designed to be 30 completely complementary to a specific sequence of interest to test for the presence of the specific sequence of interest in a sample of nucleic acids or mixture of nucleic acids. The sample of nucleic acids may be purified or unpurified, as in a sample of lysed cells or tissue.

For use in a method for detecting a nucleic acid which includes a specific sequence of interest, an oligonucleotide, whether a primer, a blocking oligonucleotide or a probe, is selected to be completely
5 complementary to the specific sequence of interest. In a particular embodiment, the specific sequence of interest is a mutation. If the specific sequence of interest is included in the nucleic acid being assessed, the oligonucleotide will hybridize to the complementary region
10 of the strand of the nucleic acid which includes the specific sequence of interest to form a homoduplex nucleic acid. MutS protein does not bind to a homoduplex nucleic acid and thus, in the case where the oligonucleotide selected is a primer, initiation of polymerization of a
15 primer extension product occurs (the desired amplification product is synthesized).

If initiation of polymerization of a primer extension product is blocked, then the specific sequence thought to be included in the nucleic acid is likely not included in
20 the nucleic acid. In this case, a nucleic acid strand and the primer have formed a heteroduplex containing a bulge loop which has been bound by MutS, indicating the presence of a mismatch or small insertion or deletion in the nucleic acid strand related to the primer. MutL protein enhances
25 binding of the MutS protein to bulge loops in the heteroduplex nucleic acid.

In the case where the oligonucleotide selected is a blocking oligonucleotide, propagation of polymerization of a primer extension product (i.e., elongation of the
30 extension product) occurs (the desired amplification product is synthesized). If propagation of polymerization of a primer extension product (i.e., elongation of the extension product) is blocked, then the specific sequence thought to be included in the nucleic acid is likely not
35 included in the nucleic acid. In this case, a nucleic acid

strand and blocking oligonucleotide have formed a heteroduplex containing a bulge loop which has been bound by MutS, indicating the presence of a mismatch or small insertion or deletion in the nucleic acid strand related to the blocking oligonucleotide. MutL protein enhances binding of the MutS protein to bulge loops in the heteroduplex nucleic acid.

In the case where the oligonucleotide selected is a probe, amplification of target nucleic acid occurs. If amplification of the nucleic acid is blocked, then the specific sequence thought to be included in the nucleic acid is likely not included in the nucleic acid. In this case, a nucleic acid strand and probe have formed a heteroduplex containing a bulge loop which has been bound by MutS, indicating the presence of a mismatch or small insertion or deletion in the nucleic acid strand related to the probe. MutL protein enhances binding of the MutS protein to bulge loops in the heteroduplex nucleic acid.

The amount of amplification product synthesized in each case is referred to herein as the amount of amplification product synthesized in a sample which comprises template nucleic acids assessed for the specific sequence of interest.

As a negative control, a mixture containing (1) a nucleic acid which does not have the specific sequence thought to be included in the template being evaluated (i.e., containing only mismatched versions of the template being evaluated) and (2) the oligonucleotide designed to be completely complementary to the specific sequence thought to be included in the template being evaluated, is maintained under (a) conditions in which primer extension is initiated in the case where the oligonucleotide is a primer or under (b) conditions in which primer extension product is elongated in the case where the oligonucleotide is a blocking oligonucleotide or under (c) conditions in

which target template is amplified in the case where the oligonucleotide is a probe. The amount of amplification product synthesized in the control is compared to the amount of amplification product synthesized in a sample
5 which comprises template nucleic acids assessed for the specific sequence of interest. If the amount of amplification product synthesized in the sample which comprises template nucleic acids assessed for the specific sequence of interest is the same as or less than the amount
10 of amplification product synthesized in the control, the specific sequence of interest is likely not included in the template nucleic acid. In the case of the opposite result (if the amount of amplification product synthesized in the sample which comprises template nucleic acids assessed for
15 the specific sequence of interest is greater than the amount of amplification product synthesized in the control), the specific sequence of interest is likely included in the template nucleic acid.

In a method for selecting against a nucleic acid
20 comprising a specific sequence, an oligonucleotide is designed to form heteroduplexes with a strand of the nucleic acid being selected against. That is, the oligonucleotide is designed to be less than completely complementary to the specific nucleotide sequence being
25 selected against (but sufficiently complementary that hybridization occurs). An oligonucleotide which is less than completely complementary to the nucleotide sequence being selected against comprises one or more nucleotide mispairings with a nucleic acid strand in the region of the
30 specific sequence being selected against when the oligonucleotide and nucleic acid strand hybridize together in that region, resulting in the formation of a bulge loop in the heteroduplex nucleic acid. An oligonucleotide which is less than completely complementary to the nucleotide

sequence being selected against can be a primer, a blocking oligonucleotide or a probe.

Oligonucleotides may be prepared using any suitable method, such as, for example, the phosphotriester and
5 phosphodiester methods, or automated embodiments thereof. In one such automated embodiment diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage et al., *Tetrahedron Letters* 22: 1859-1962 (1981). Oligonucleotides can also be synthesized by
10 phosphoramidite chemistry in a Milligene 8750 DNA synthesizer according to the manufacturer's specification. One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066. It is also possible to use a primer which has been isolated
15 from a biological source (such as a restriction endonuclease digest).

The thermostable proteins of the present invention which enhance binding of thermostable mismatch binding proteins to bulge loops in a heteroduplex nucleic acid may
20 be used with thermostable mismatch binding proteins in any methods of amplification of nucleic acids to improve fidelity or to improve allele-specific amplification. For example, the binding of thermostable mismatch binding proteins such as MutS proteins to DNA containing
25 replication errors caused by misincorporation by a DNA polymerase, can improve the fidelity of the sequence of DNA in amplification methods, and has applications, for example, in the cloning of a true copy of genomic DNA. Addition of a thermostable protein that enhances binding of
30 thermostable mismatch binding proteins to bulge loops can improve this result.

Where searching or assaying for DNA of a specific sequence among a mixture of many DNA molecules, methods of DNA amplification rely on the specificity of primer
35 oligonucleotides annealing to a perfectly matched

complementary strand in the template DNA. The addition to amplification reactions of a thermostable mismatch binding protein that binds to bulge loops formed when primer-template mismatches occur, and that prevents extension from the primer, can eliminate or greatly reduce the amplification from sites at which the primer-template complementarity is less than perfect. Addition of a thermostable protein that enhances binding of thermostable mismatch binding proteins to bulge loops can improve this result. Variations on this method can be used to detect particular nucleic acid sequences that occur in cancer and in various genetic diseases.

The methods of the present invention are based on known methods of amplification of nucleic acids. Reagents used in the methods can be added sequentially or simultaneously. If a method of strand separation, such as heat, is employed which will inactivate the inducing agent, as in the case of a heat-labile enzyme, then it is necessary to replenish the inducing agent after every strand separation step.

PCR is an example of an amplification technique. PCR refers to an amplification technique where a pair of primers (one primary and one secondary) is employed in excess to hybridize at the outside ends of complementary strands of the target nucleic acid. The primers are each extended by a polymerase using the target nucleic acid as a template. The extension products become target sequences themselves, following dissociation from the original target strand. New primers are then hybridized and extended by a polymerase, and the cycle is repeated to increase geometrically the number of target sequence molecules. PCR is described further in U.S. Patent No. 4,683,195; U.S. Patent No. 4,683,202; U.S. Patent No. 4,800,159; and U.S. Patent No. 4,965,188. Many variations of PCR are known.

(See, e.g., Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, John Wiley & Sons Inc., New York, 1994).

LCR is another example of an amplification technique. LCR refers to an amplification technique where two primary
5 (first and second probes) and two secondary (third and fourth) probes are employed in excess. The first probe hybridizes to a first segment of the target strand and the second probe hybridizes to a second segment of the target strand, the first and second segments being contiguous so
10 that the primary probes abut one another in 5' phosphate-3' hydroxyl relationship and so that a ligase can covalently fuse or ligate the two probes into a fused product. In addition, a third (secondary) probe can hybridize to the first probe and a fourth (secondary) probe can hybridize to
15 the second probe in a similar abutting fashion. If the target is initially double stranded, the secondary probes will also hybridize to the target complement in the first instance. Once the fused strand of primary probes is separated from the target strand, it will hybridize with
20 the third and fourth probes which can be ligated to form a complementary, secondary fused product. The fused products are functionally equivalent to either the target or its complement. By repeated cycles of hybridization and ligation, amplification of the target sequence is achieved.
25 This technique is described further in, for example, EP-A-320 308 and European Application No. 0 439 182 A2 (published July 31, 1991).

The methods herein may be used to enable detection and/or characterization of particular nucleic acid
30 sequences associated with infectious diseases, genetic disorders or cellular disorders such as cancer, e.g., oncogenes. For example, the methods herein may be used to detect early mutations in cells in sputum, feces, urine, or blood which predispose cells to progress to malignancy.
35 The methods herein may be used in metastasis (e.g., for

screening lymph nodes for cells containing the same mutations found in a primary solid tumor or for detecting reoccurrence of a hematological disease).

One embodiment of the invention relates to detecting
5 nucleic acids which include a specific nucleotide sequence comprising combining a thermostable mismatch binding protein which binds specifically to bulge loops in a heteroduplex nucleic acid, a thermostable protein that enhances binding of the thermostable mismatch binding
10 protein to the bulge loops, and an amplification reaction mixture, to produce a test combination. The individual components of an amplification reaction mixture can each be added, together or separately (e.g., individually), in any order, prior to, subsequent to or simultaneously with the
15 thermostable mismatch binding protein which binds specifically to bulge loops in a heteroduplex nucleic acid, and/or the thermostable protein that enhances binding of the thermostable mismatch binding protein to the bulge loops. The resulting test combination is maintained under
20 conditions appropriate for nucleic acid amplification to occur (i.e., synthesis of extension product). The amount of extension product synthesized in the test combination is determined and compared with the amount of product synthesized in a corresponding negative control (the
25 control amount) to determine if the specific nucleotide sequence suspected of being present in the nucleic acids being assessed is present. If the amount of product synthesized in the test combination is the same as or less than the amount of product synthesized in the corresponding
30 negative control, then the nucleic acids being assessed do not include the specific nucleotide sequence. If the amount of product synthesized in the test combination is greater than the amount of product synthesized in the corresponding control, then the nucleic acids being
35 assessed include the specific nucleotide sequence. In a

particular embodiment, the specific nucleotide sequence is a mutation.

In a particular embodiment, the components of an amplification reaction mixture include (1) a nucleic acid
5 to be assessed for a specific nucleotide sequence of interest; (2) four different nucleoside triphosphates; (3) two oligonucleotide primers where each primer is selected to be complementary to different strands of the nucleic acid which includes the specific nucleotide sequence of
10 interest such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer, at a temperature which promotes hybridization of each primer to its complementary strand; (4) a blocking
15 oligonucleotide completely complementary to the specific nucleotide sequence of interest; (5) a thermostable enzyme which catalyzes combination of the nucleoside triphosphates to form primer extension products complementary to each strand of the nucleic acid which includes the specific
20 nucleotide sequence of interest; and (6) an amplification buffer suitable for the activity of the enzyme. Thus, for example, one or more of the different nucleoside triphosphates can be added prior to, subsequent to or simultaneously with the thermostable mismatch binding
25 protein which binds specifically to bulge loops in a heteroduplex nucleic acid and/or the thermostable protein that enhances binding of the thermostable mismatch binding protein to the bulge loops. One or more of the primers can be added prior to, subsequent to or simultaneously with one
30 or more of the different nucleoside triphosphates, the thermostable mismatch binding protein and/or the thermostable protein that enhances binding of the thermostable mismatch binding protein to the bulge loops. Similarly, the blocking oligonucleotide, the thermostable
35 enzyme, the nucleic acid to be assessed for the nucleotide

sequence of interest and/or the amplification buffer can each be added prior to, subsequent to or simultaneously with one or more of the different nucleoside triphosphates, one or more of the primer, the thermostable mismatch binding protein and/or the thermostable protein that enhances binding of the thermostable mismatch binding protein to the bulge loops. The blocking oligonucleotide, the thermostable enzyme, the nucleic acid to be assessed for the nucleotide sequence of interest, and the amplification buffer can also be added in any order relative to each other. In another embodiment, the amplification reaction mixture further includes a second blocking oligonucleotide designed to be completely complementary to the complementary strand of the nucleotide sequence of interest. Complementary overlap between the second blocking oligonucleotide and the first blocking oligonucleotide (the blocking oligonucleotide designed to be completely complementary to the specific nucleotide sequence of interest) should be minimized to avoid the stable annealing of the oligonucleotides to each other.

In a further embodiment, the components of an amplification reaction mixture include (1) a nucleic acid to be assessed for a specific nucleotide sequence of interest; (2) four different nucleoside triphosphates; (3) two oligonucleotide primers where each primer is selected to be complementary to different strands of the nucleic acid which includes the specific nucleotide sequence of interest, with one primer completely complementary to the nucleotide sequence of interest, such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer, at a temperature which promotes hybridization of each primer to its complementary strand; (4) a thermostable enzyme which catalyzes combination of the nucleoside triphosphates to

form primer extension products complementary to each strand of the nucleic acid which includes the specific nucleotide sequence of interest; and (5) an amplification buffer suitable for the activity of the enzyme. In a particular
5 embodiment, the amplification reaction mixture further include a blocking oligonucleotide completely complementary to the complementary strand of the specific nucleotide sequence of interest.

In another embodiment, the components of an
10 amplification reaction mixture include (1) a nucleic acid to be assessed for a specific nucleotide sequence of interest; (2) four oligonucleotide probes, two primary and two secondary probes, with one primary probe completely complementary to the nucleotide sequence of interest and
15 one secondary probe completely complementary to the complementary strand of the nucleotide sequence of interest; (3) a thermostable enzyme which catalyzes fusion of oligonucleotide probes to form amplified products complementary to each strand of the nucleic acid which
20 includes the specific nucleotide sequence of interest; and (4) an amplification buffer suitable for the activity of the enzyme. In a particular embodiment, one of the probes which is completely complementary to the nucleotide sequence of interest is omitted.

25 The three embodiments describing components of the amplification reaction mixture are not intended to be limiting in any way. In each particular embodiment, the amplification reaction mixture can further include additional components, such as, for example, components
30 which enhance the activity of thermostable enzymes to catalyze combination of nucleoside triphosphates to form primer extension products or components which enhance and/or improve the amplification reaction and/or the utility of the amplification procedure. The components of
35 an amplification reaction mixture and amplification

conditions depend upon the particular amplification procedure being employed and can be determined from readily available sources. See, for example, Ausubel et al., *Current Protocols In Molecular Biology*, John Wiley & Sons, New York, 1994; Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York, 1989; U.S. Patent No. 4,683,195; U.S. Patent No. 4,683,202; U.S. Patent No. 4,800,159; U.S. Patent No. 4,965,188; European Patent Application No. 0 416 677 A1 (published March 13, 1991); Holland et al., *Proc. Natl. Acad. Sci. USA* 88:7276-7280 (1991); Livak et al., *Nat. Genet.* 9:341-342 (1995); Saiki et al., *Proc. Natl. Acad. Sci. USA* 86:6230-6234 (1989); Nickerson et al., *Proc. Natl. Acad. Sci. USA* 87:8923-8927 (1990); Kwok et al., *Nucleic Acids Res.* 18:999-1005 (1990); Tada et al., *Cancer Res.* 53:2472-2474 (1993); Bottema et al., *Methods Enzymol.* 218:388-402 (1993); Wiedmann et al., *PCR Methods & Applications* 3:S51-64 (1994); Felley-Bosco et al., *Nucleic Acids Res.* 19:2913-2919 (1991); Cha et al., *PCR. Methods. Appl.* 2:14-20 (1992); Hruban et al., *Am. J. Pathol.* 143:545-554 (1993); Sidransky et al., *Science* 256:102-105 (1992); and Hsu et al., *Carcinogenesis* 15:1657-1662 (1994). The components of an amplification mixture further depend on whether the specific nucleotide sequence of interest is in, for example, a region of high GC content or a region of high AT content.

Oligonucleotide-template hybridizations are more stable in regions of high GC content than in regions of high AT content. Thus, if the specific nucleotide sequence of interest is in, for example, a region of high AT content, one embodiment of the invention can be to select two oligonucleotide primers to be complementary to different strands of a nucleic acid which includes the specific nucleotide sequence of interest to hybridize therewith and a blocking oligonucleotide designed to be

completely complementary to the specific nucleotide sequence of interest. If the specific nucleotide sequence of interest is in, for example, a region of high GC content, one embodiment of the invention can be to select
5 primers to be complementary to different strands of a nucleic acid which includes the specific nucleotide sequence of interest to hybridize therewith, with one primer completely complementary to the specific nucleotide sequence of interest. In a particular embodiment, the
10 specific nucleotide sequence of interest is a mutation.

As discussed above, oligonucleotides which are designed to be completely complementary to the specific nucleotide sequence of interest can be designed for use in pairs, one oligonucleotide to anneal to and block the
15 amplification of each complementary strand of the template, for example, in a control sample (i.e., in a sample of nucleic acids known to not include the specific nucleotide sequence of interest). The oligonucleotides can also be designed for use as a single oligonucleotide, annealing to
20 and blocking the amplification of one strand of the template, for example, in a control sample (i.e., in a sample of nucleic acids known to not include the specific nucleotide sequence of interest). If oligonucleotides are designed for use in pairs, complementary overlap between
25 the oligonucleotides in a pair should be minimized to avoid the stable annealing of the oligonucleotides to each other.

Stabilizers can be included in the methods of the present invention. As used herein, for example, stabilizers increase the lifetime of a thermostable bulge
30 loop-binding protein-heteroduplex nucleic acid complexes. For example, stabilizers herein increase the lifetime of MutS-heteroduplex nucleic acid complexes. A MutS-heteroduplex nucleic acid complex is a complex formed when MutS is bound to a bulge loop in a heteroduplex nucleic
35 acid. ATP γ S is an example of a stabilizer.

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Other proteins which may be included in the methods of the present invention include those associated with nucleic acid strand discrimination (e.g., thermostable MutH or homologs thereof), those that enhance the activity of stabilizers to increase the lifetime of a thermostable bulge loop-binding protein-heteroduplex nucleic acid complexes, and those that enhance the activity of thermostable enzymes to catalyze combination of nucleoside triphosphates to form primer extension products.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLES

Example 1 Genomic DNA, Plasmids, Nucleotides and Enzymes

All DNA manipulations used standard techniques and procedures (Sambrook, J. et al., *Molecular Cloning, A Laboratory Manual*, 2nd edition, Cold Spring Harbor: Cold Spring Harbor University Press (1989)). Genomic DNAs of *Thermotoga maritima* (Tma) and *Aquifex pyrophilus* (Apy) (Burggraf, S. et al., *System. Appl. Microbiol.* 15: 352-356 (1992)), both from cells supplied by Professor Karl Stetter, Universität Regensburg, were extracted for use as PCR templates and for Southern blots. Plasmids employed for cloning and expression were pUC19, pDG160/pDG182/pDG184 (Lawyer, F.C. et al., *PCR. Methods. Appl.* 2: 275-287 (1993)) and pET16b (Novagen, Inc.), which were grown in *E. coli* DH5 α , DG116 (Lawyer, F.C. et al., *PCR. Methods. Appl.* 2: 275-287 (1993)) and BL21(DE3), respectively. All absorbance spectra were determined using a Hewlett-Packard diode array spectrophotometer equipped with a peltier temperature controller. Concentrations of DNA and primers were determined by using 50 and 36 $\mu\text{g ml}^{-1}$ A_{260}^{-1} ,

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respectively, as conversion factors. Deoxynucleoside triphosphates were purchased from Boehringer-Mannheim. [α - 35 S]dATP and [γ - 32 P] ATP were purchased from NEN/DuPont. *E. coli* MutS protein was provided by U.S. Biochemical, Inc.

5 UDG (uracyl DNA glycosylase, uracil N-glycosylase) was purchased from BRL, Inc. and used according to the manufacturer's instructions. Amplitaq DNA Polymerase, purchased from Perkin-Elmer, and native Taq polymerase, purchased from several suppliers, were used in the buffer

10 supplied by the manufacturer. Restriction endonucleases, T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs and used as recommended by the manufacturer. Simultaneous reactions with two or more restriction endonucleases were carried out in New England

15 Biolabs NEB3 buffer. Simultaneous reactions with restriction endonucleases and T4 DNA ligase were carried out in the same buffer supplemented with 1 mM ATP.

Example 2 Oligodeoxynucleotides

All synthetic oligodeoxynucleotide primers for PCR and

20 sequencing were synthesized on automated instruments using standard phosphoramidite chemistry.

Degenerate primers were constructed based on the following rules. First, the corresponding amino acid sequences should be identical in representative Gram-

25 positive (e.g. *E. coli*) and Gram-negative organisms (e.g. *S. pneumoniae*) and should not be a common motif in unrelated proteins. For example, sequences satisfying this rule include MGDIFYE, PNMGGK and FATHY located at positions 19, 614 and 725 in *E. coli* MutS, respectively. Similarly

30 conserved sequences include IAAGEV and GFRGEA located at positions 14 and 93 in *E. coli* MutL, respectively. Second, the length of the sequence to be amplified should be kept as short as possible, consistent with obtaining an informative sequence in the PCR product, in order to

maximize specific PCR amplification and minimize the likelihood of occurrence of *EcoRI*, *BglII* or *BamHI* sites which could interfere with subsequent cloning. Thus, degenerate primers based on MGDFYE were not used for the
5 initial *mutS* amplifications. Third, the degeneracy should be minimized by taking advantage of codon usage whenever possible. For example, in contrast to *Thermus* species, both *Apy* and *Tma* use AGR instead of CGN arginine codons more than 90% of the time. Fourth, except for the use of
10 complete degeneracy in the last 5 nucleotides at the 3' end of a primer where a mismatch may have a deleterious effect on PCR, the following substitutions were made: G for R, C for Y, G/C for N. Reduced primer degeneracy increases primer template hybridization rates which can limit
15 degenerate PCR (Wetmur, J.G. and Sninsky, J.J., In: PCR Strategies, Innis, M.A. et al., Eds., Academic Press, San Diego, pp. 69-83, 1995).

Primer construction is illustrated for *mutS* cloning. The initial degenerate sense primer
20 5' GCGGAATTCC (G/C) AACATGGG (G/C) GG (A/C/G/T) AA 3' (SEQ ID NO:19) and antisense primer
5' GCGAGATCTAAGTAGTG (G/C) GT (A/C/G/T) GC (G/A) AA 3' (SEQ ID NO:20), corresponding to amino acids 615-620 and 725-729 in *E. coli* MutS, were used for cloning a fragment of the *Apy*
25 and *Tma* *mutS* genes. *EcoRI* (GAATTC) and *BglII* (AGATCT) recognition sequences are underlined.

Apy- and *Tma*-specific antisense primers,
5' GCGAGATCTCACCTGTCTTATGTAGCTCGA 3' (SEQ ID NO:21) and
5' GCGAGATCTCATCTCGACAAG-GAACGTACT 3' (SEQ ID NO:22),
30 respectively, were employed together with a third degenerate sense primer,
5' GCGGAATTCATGGGGGA (C/T) TT (C/T) TA (C/T) GA 3' (SEQ ID NO:23), corresponding to amino acids 33-38 in *E. coli* MutS. Specific inverse primers for use with near the 5' end of
35 the known sequence were

5' GCGGAATTCGGGAAAGGATTCCTCATGTTTCG 3' (SEQ ID NO:24) and
5' GCGAGATCTCCTTTCCA-GCGGGTCTTGAAG 3' (SEQ ID NO:25) for
Apy and 5' GCGGAATTCGGGCATCCCGTACCACTCGC 3' (SEQ ID NO:26)
and 5' GCGAGATCTGGAGCGTCCCTGCCCTTCTTG 3' (SEQ ID NO:27) for
5 Tma.

Specific inverse primers for use with near the 3' end
of the known sequence were

5' GCGGAATTCCTCAACCTTCATGAA-CGAGATG 3' (SEQ ID NO:28) and
5' GCGAGATCTCGAGCCTATTCTCATGAATAT 3' (SEQ ID NO:29) for Apy
10 and 5' GCGGAATTCGAGGTGGGAAGAGGTACAAGC 3' (SEQ ID NO:30) and
5' GCGAGATCTCATCTCGACAAG-GAACGTACT 3' (SEQ ID NO:31) for
Tma.

Additional sequencing primers lacking the GCG cap and
restriction endonuclease sites were synthesized as
15 required. These species-specific oligodeoxynucleotides
were employed for Southern hybridization.

PCR primers for cloning Tma *mutS* genes into pDG160
were 5' GCGAAGCTTATGAAGGTAAGTCCCTCATG 3' (SEQ ID NO:32)
and 5' GCGGGATCCAC-GCATCGATACTGGTTAAAA 3' (SEQ ID NO:33),
20 where the *Bam*HI and *Hind*III sites are underlined and the
initiation codon in the forward primer is shown in bold
italics.

PCR primers for cloning Apy *mutS* genes into pDG182 and
pDG184 and pET16b were
25 5' GCGCCATGGGAAAAGAGGA-GAAAGAGCTCA 3' (SEQ ID NO:34) and
5' GCGAGATCTGATACTCCAGAGGTATTACAA 3' (SEQ ID NO:35) where
the *Nco*I, which contains the initiation codon, and *Bgl*II
sites are underlined.

Example 3 DNA Amplification

30 PCR amplifications were carried out in a
USA/Scientific Gene Machine II or an Ericomp PowerBlock
System with DNA templates in 50-100 μ l containing 1 μ M of
each primer, 10 mM Tris-HCl buffer, pH 8.3, 50 mM KCl, 25-
50 units/ml Taq DNA polymerase, and 200 μ M of each dNTP

(Saiki, R.K. et al., *Science* 239: 487-491 (1988)). Typically, simultaneous reactions were initiated by addition of a $MgCl_2$ solution to Mg^{++} -free PCR mixtures at $>80^\circ C$ to yield final concentrations of 0.8-2 mM followed by
5 denaturation for 30 sec at $95^\circ C$. When using degenerate primers and 50 ng genomic DNA template, the first 5 cycles employed a 30 sec annealing step at $45^\circ C$ followed by a 2 min ramp to $72^\circ C$ before denaturation. An additional 30-35
10 cycles were carried out with a $55^\circ C$ annealing temperature. For inverse PCR (Ochman, H. et al., In *PCR Protocols. A Guide to Methods and Applications*, Innis, M.A. et al., Eds. (San Diego: Academic Press, Inc) pp. 219-227 (1990)),
genomic DNA was digested to completion with a restriction endonuclease leaving a 3' or 5' 4-base overhang, phenol
15 extracted, and ligated overnight at a DNA concentration of less than 50 $\mu g/ml$. When using unique direct or inverse PCR primers, templates of 50 ng genomic DNA or circularized genomic DNA, respectively, were employed, and the first 5 cycles were omitted.

20 Example 4 Cloning, Sequencing and Southern
 Hybridization

Products of PCR amplifications were phenol extracted to remove Taq polymerase and filtered on Millipore Ultrafree-MC 30,000 NMWL filter units to remove primers.
25 PCR products with *Bgl*II cloning sites were cloned into pUC19 by simultaneous digestion of vector and insert with *Bgl*II, *Bam*HI, and *Eco*RI, heat inactivation, ligation, and re-digestion with *Bam*HI to destroy religated vectors without inserts. Inserts in pUC19, pDG160, pDG182, pDG184
30 and pET16b were sequenced in both orientations using insert-specific and vector-specific oligodeoxynucleotide primers with the Sequenase DNA Sequencing Kit (U.S. Biochemicals, Inc.) or by cycle sequencing with Taq DNA polymerase using either ^{32}P -labeled primers (Gibco-BRL kit)

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or fluorescent dideoxy terminators on an Applied Biosystems Automated DNA Sequencer. Southern hybridizations of restriction endonuclease-cleaved genomic DNAs were carried out with oligodeoxynucleotides labeled with ^{32}P using T4 polynucleotide kinase. The genomic DNAs and restriction endonucleases were (1) Apy, none; (2) Apy, *HindIII*; (3) Apy, *SacI*; (4) Tma, *BglIII*; (5) Tma, *HindIII*; (6) Tth, *BamHI*; (7) Tth, *SacI*; (8) Tth, none; (9) Taq, partial *SacI*; (10) Taq, *SacI*.

10 Example 5 Cloning and Sequence Analysis of *mutS* and *mutL* Genes From *Aquifex pyrophilus* and *Thermotoga maritima*

The cloning of the *mutS* and *mutL* genes from *Aquifex pyrophilus* and *Thermotoga maritima* was accomplished without library construction using the same approach employed for the cloning of four thermophilic or hyperthermophilic RecA proteins (Wetmur, J.G. et al., *J. Biol. Chem.* 269: 25928-25935 (1994)). Fragments of Apy and Tma *mutS* and *mutL* were amplified using a single set of degenerate PCR primers for each of the genes. Each primer began with GCG, followed by either an *EcoRI* or a *BglIII* site, and followed by a degenerate nucleotide sequence.

The amplifications yielded unique products of the predicted length, which were cloned into pUC19 and sequenced using vector-specific primers. Although significant variation was observed for the translated sequence between the primers, Apy and Tma *MutS* and Apy and Tma *MutL* sequences were unmistakably those of *MutS* and *MutL* proteins, respectively. Longer (1.8 kb) fragments of both *mutS* genes were obtained using a unique antisense primer based on the newly acquired sequence and a degenerate sense primer based on the conserved MGDFYE sequence.

Unique inverse PCR cloning primers were synthesized corresponding to sequences near the 5' and 3' ends of each

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of the fragments and employed for amplifying genomic DNA circularized using various restriction endonucleases and DNA ligase. Southern blots were tested using sequence-specific oligodeoxynucleotides sequentially as probes. The
5 Apy and Tma probes bound with equal efficiency only to Apy and Tma genomic DNA, respectively, but not to the DNA from several other species. These binding specificities demonstrated that the sequences amplified by PCR were
10 were iterated as necessary until the sequences extended 5' from the initiation codon and well beyond the termination codon. To be certain that the sequences to be incorporated into the 5'-PCR expression primers accurately reflected the genomic sequence, the 5' sequence was verified by cycle
15 sequencing.

The *mutS* and *mutL* genes from both of the hyperthermophiles were amplified using expression primers. Examples of expression primers are provided in Example 2. Products of several independent PCR reactions were digested
20 with the appropriate restriction endonucleases and ligated into expression vectors. Clones which expressed a thermostable MutS or MutL were completely sequenced. The *mutS* and *mutL* amino acid sequences shown in Figures 10 and 11, respectively, were determined to be authentic because
25 they were identical in at least two independently-derived clones. The guanine plus cytosine content (G+C%) of all four complete sequences was approximately 47%, as expected.

A TFASTA analysis comparing the *E. coli* MutS amino acid sequence with the translated Apy and Tma MutS
30 sequences is depicted in Figure 10. The numbers refer to amino acid positions in *E. coli* MutS. The TFASTA analysis depicted in Figure 10 for Apy and *E. coli* (853 amino acids) MutS shows 36% identity in 792 amino acids overlap with length differences at the N- and C-termini of only 2 and 6
35 amino acids, respectively. The TFASTA analysis depicted in

Figure 10 for Tma and *E. coli* MutS shows a similar 37% identity in 783 amino acids overlap. However, Tma MutS showed significant variation at both the N- and C-termini. The analysis of the ends is outlined in Figure 12.

5 Following the last in-frame stop codon (TGA), the first ATG in Tma *mutS* matched the ATG at *E. coli mutS* codon 14. However, there were conserved threonine and proline codons at 3 and 2 positions upstream from this ATG in *E. coli*, Apy and Tma. Further examination of this upstream region
10 revealed three valine codons (GTN). The most distal of these codons appeared to occur deep in the open reading frame of an upstream gene (termination TGA). The other two codons followed 5 and 11 nt after a sequence matching in 9 of 10 positions the 3' end of Tma 16S ribosomal RNA, 3'
15 UUCCuCCACU 5' (Benson, D. et al., *Nucleic Acids Res.* 21: 2963-2965 (1993)). Because the 5 nt spacing separated the valine codon from the presumptive ribosome binding site by the optimal spacing, this codon was taken to be the initiation codon and was incorporated as ATG into the sense
20 expression primer. This N-terminal was thus 7, rather than 13, and 5 amino acids shorter than *E. coli* and Apy MutS, respectively.

A PILEUP analysis comparing the MutL homolog *S. pneumoniae* (Spn) HexB and *E. coli* (Eco) MutL amino acid
25 sequences with the coding sequences of Apy and Tma MutL is depicted in Figure 11. The positions of the N-terminal amino acids only varied by 1 amino acid. The initiation codon for Apy MutL was again a GTN codon and was incorporated as ATG into the sense expression primer. Only
30 the N-terminal half of MutL proteins is conserved. TFASTA analysis with the first 200 amino acids of the MutL proteins showed that whereas Eco and Spn proteins were 50% identical, Apy MutL was 39, 42 and 45% identical to Spn HexB, Tma MutL and Eco MutL, respectively, and Tma MutL was
35 43% identical to both Eco MutL and Spn HexB.

The C-terminus of Tma MutS was 35 and 41 amino acids shorter than *E. coli* and Apy MutS, respectively. An investigation of the downstream flanking sequence revealed an open reading frame in reverse orientation which overlapped Tma MutS by 8 amino acids and which could encode a protein similar to that encoded by the D-ribulose-5-phosphate epimerase gene of *Alcaligenes eutrophus* and the *dod* gene of *Serratia marcescens*.

The major surprise came at the C-termini of the MutL proteins. Although this region of MutL is not generally conserved, the sizes of Eco MutL (615 amino acids), Spn HexB (649 amino acids) and other bacterial MutL sequences in Genbank are approximately the same. Tma and Apy MutL contain only 511 and 426 amino acids, respectively. The authenticity of the C-termini (e.g. no introns) was bolstered by the observation of a conserved CPHGRP(I/V) sequence 15-30 amino acids from the C-termini of the Apy MutL, Tma MutL and Spn HexB.

Cloning and sequence analysis of thermophilic *mutS* genes are also described in U.S. Application No. 08/468,558 (filed June 6, 1995) and International Application No. PCT/US96/08677 (filed June 4, 1996). See also International Publication No. WO 96/39525 (published December 12, 1996).

Example 6 Phylogenetic Analysis of Apy and Tma MutS and MutL Protein Sequences

Nucleic acid and protein sequence analyses were carried out using programs in GCG (Devereux, J. et al., *Nucleic Acids Res.* 12: 387-395 (1984)). Because the guanine plus cytosine content of the hyperthermophiles was about 47%, amino acid substitutions were not expected to reflect codon bias. TFASTA analysis of both MutS and MutL proteins and their homologs indicated that the amino acid sequences of the hyperthermophilic eubacteria, Gram-

negative bacteria and Gram-positive bacteria were equally divergent, as had previously been observed using other proteins or 16S rRNA (Wetmur, J.G. et al., *J. Biol. Chem.* 269: 25928-25935 (1994); Burggraf, S. et al., *System. Appl. Microbiol.* 15: 352-356 (1992)).

5 Using PILEUP, the newly determined sequences of the thermophilic MutS and MutL proteins were aligned with related sequences in Genbank (Benson, D. et al., *Nucleic Acids Res.* 21: 2963-2965 (1993)) for at least two Gram-
10 negative and two Gram-positive mesophilic bacteria and additional eukaryotic MutS or MutL homolog sequences. The multiple alignments were truncated to include only amino acids corresponding to 8 - 794 of *E. coli* MutS and 1-199 of
15 *E. coli* MutL prior to analysis using PHYLIP (Phylogeny Inference Package) version 3.5c (Felsenstein, J., *Cladistics* 5: 164-166 (1989)). Pairwise distances between amino acids in the MutS and MutS homolog sequences were calculated using PROTDIST with the Dayhoff PAM matrix. Unrooted trees, calculated using FITCH with global
20 rearrangement and jumbling before plotting with DRAWTREE, revealed Apy MutS, Tma MutS and the set of all mesophilic eubacterial MutS homologs to be equally divergent. The same result was observed for MutL.

Example 7 Expression of Apy and Tma MutS and MutL
25 Proteins

Expression primers were a 5'-PCR primer containing a GCG cap, a restriction endonuclease site, an initiation ATG and the next 20 nucleotides of the coding sequence and a
30 3'-PCR primer containing a GCG cap, a second restriction endonuclease site and 21 nucleotides antisense to the downstream flanking sequence. PCR products from both species were ligated into pDG160/pDG182/pDG184 (APy) (Lawyer, F.C. et al., *PCR. Methods. Appl.* 2: 275-287 (1993)) or pET16b (Novagen, Inc.) and electroporated into

E. coli DG116 (Lawyer, F.C. et al., *PCR. Methods. Appl.* 2: 275-287 (1993)) cells expressing the pLySS plasmid (Novagen, Inc) or BL21(DE3), respectively. The pLySS plasmid permits cell lysis by freeze-thaw.

5 Examples of PCR expression primers include

5' GCGAAGCTT***TATGAAGGTA***ACTCCCCTCATG 3' (SEQ ID NO:32) and

5' GCGGGATCCACGCATCGATACTGGTTAAAA 3' (SEQ ID NO:33) for

cloning *Tma mutS* genes into pDG160, where the *Bam*HI and *Hind*III sites are underlined and the initiation codon in

10 the forward primer is shown in bold italics, and

5' GCGCCATGGGAAAAGAGGAGAAAGAGCTCA 3' (SEQ ID NO:34) and

5' GCGAGATCTGATACTCCAGAGGTATTACAA 3' (SEQ ID NO:35) for

cloning *Apy mutS* genes into pDG182, pDG184 and pET16b, where the *Nco*I site, which contains the initiation codon,

15 and *Bgl*II sites are underlined.

E. coli DG116 colonies derived from independent amplification reactions were grown overnight at 30°C in LB-AMP-chloramphenicol, diluted 1/100 into the same medium and grown to A_{600} approximately equal to 0.75, induced at 42°C

20 for 15 min, grown for an additional 3-5 hrs at 39°C, and collected by centrifugation for 15 min at 6,000 g. *E. coli* BL21(DE3) colonies were grown overnight at 37°C in LB-AMP-chloramphenicol, diluted 1/100 into the same medium and grown to A_{600} approximately equal to 0.75, induced with 1
25 mM IPTG, grown for an additional 5-12 hrs, and collected by centrifugation for 15 min at 6,000 g.

The pellets were resuspended in 300 μ l 50 mM Tris-HCl, 1 mM PMSF, 1 mM DTT and 10 mM EDTA, pH 8 for each 100 ml of culture and subjected to 3 cycles of freezing in dry-ice
30 ethanol and thawing at 37°C. Following sonication on ice to reduce the viscosity and centrifugation to remove cell debris, the samples were transferred to a new tube, made 0.3 M $(\text{NH}_4)_2\text{SO}_4$ by addition of 3 M stock, made 0.75% polyethylenimine (PEI) by addition of a neutralized 10%
35 stock to precipitate DNA, heated to 75°C for 15 min to

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denature thermolabile proteins, placed on ice for 30 min to aggregate the denatured proteins, cleared of DNA and denatured proteins by centrifugation, transferred to a new tube and frozen at -20°C (optional). The partially

5 purified MutS or MutL products were assayed for the presence of a thermostable protein of the correct size by SDS-PAGE. The presence of MutS or MutL bands was shown to depend upon the presence of the insert in the plasmid and induction by heat or IPTG.

10 Two purification schemes have been employed. In the first scheme, crude MutS or MutL, approximately 1 ml per 250 ml culture, was loaded onto a 1 ml HiTrap Q anion exchange column (Pharmacia), repeatedly washed with buffer and eluted with stepwise increases of NaCl (from about
15 0.1 M - 2.0 M) in the same buffer. The eluate was loaded onto a 1 ml HiTrap SP anion exchange column (Pharmacia) or HiTrap blue affinity column (Pharmacia). Columns were washed extensively with stepwise increases of 0.5 M NaCl plus buffer and eluted in 1-2 M NaCl or 1-2 M guanidine
20 HCl, respectively, in the same buffer. After dialysis and concentration using Centricon-30 (Amicon), protein concentrations were determined and compared with complete absorbance spectra to determine an extinction coefficient and to verify removal of nucleic acids. Purification from
25 other proteins was verified by examination of overloaded SDS-PAGE. It is important to note that BL21 is not an *endoA* strain, so care must be exercised to assure removal of endonuclease I (non-specific dsDNA specific). Endonuclease I was verified to be thermostable and
30 thermoactive.

In the second purification scheme, crude MutS or MutL was separated by BU hydrophobic chromatography on a PerSeptive Biosystems BioCAD SPRINT perfusion chromatography system. Again, removal of all nucleic acids
35 was verified by an A_{280}/A_{260} ratio greater than 1.5.

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The thermostable MutS proteins showed a single band by SDS-PAGE. The overall yield of the thermostable MutS proteins from various preparations was approximately 0.2-0.3 mg/10¹¹ cells, corresponding to 2.5-4% of the initial protein content of the cells.

Purification of Apy MutL using the first purification scheme led to a mixture of two polypeptides, one at 75 kd and one at 45 kd. Of greatest importance, this MutL preparation was active in the TaqMan assay described in Example 8. The 75 kd protein, which matched *E. coli* MutL in size, was initially purified. This purified 75 kd protein was not active in the TaqMan assay. The 45 kd protein was subsequently purified and shown to be Apy MutL. One explanation for the lower yield of Apy MutL (about 0.5-1% of initial protein), compared to the yield of Apy MutS, may be the long 3' untranslated sequence. A similar yield was obtained with Tma MutL. Tailored *mutL* genes, recloned into pD6182, have led to improved yields.

Example 8 Allele-Specific PCR

In one experiment, two plasmid templates were mixed and used in 50 μ l PCR reactions. In pUC19GC, the *Bam*HI site in the pUC19 sequence GGGGATCCTC (SEQ ID NO:10) was modified to substitute a C for the first T to yield GGGGACCCTC with a new *Ava*II site. In pUC19 Δ 3, a T and two Cs were inserted into the pUC19GC polylinker sequence GGGGACCCTC to yield GGGGATCCCCCTC (SEQ ID NO:13) and reconstitute the *Bam*HI site. The PCR primers were located at the pUC19 *Pvu*II sites. A TaqMan 28-mer oligonucleotide, terminating in a 3'P to prevent extension, matched pUC19GC completely and mismatched pUC19 Δ 3 eight nucleotides from its 5' end. The results of one TaqMan experiment using Taq Stoffel fragment DNA polymerase is shown in the Table.

TABLE

Sample	1	2	3	4	5
pUC19Δ3 (pg)	50	50	50	50	50
pUC19GC (pg)	50	2.5	0.25	0.025	0.0025
5 % Cleavage of PCR product with MutS (1 μM) and no MutL (ND = None Detected)					
AvaII (pUC19GC)	50	5	ND	ND	ND
BamHI (pUC19Δ3)	50	95	100	100	100
10 % Cleavage of PCR product with MutS (1 μM) and MutL (0.2 μM)					
AvaII (pUC19GC)	100	100	95	70	10
BamHI (pUC19Δ3)	ND	ND	5	30	90

Equivalents

Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: THERMOSTABLE MUTL GENES AND PROTEINS AND
USES THEREFOR

(iii) NUMBER OF SEQUENCES: 45

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/676,444
(B) FILING DATE: 05-JUL-1996

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2568 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..2565

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG GGA AAA GAG GAG AAA GAG CTC ACC CCC ATG CTC GCC CAG TAT CAC	48
Met Gly Lys Glu Glu Lys Glu Leu Thr Pro Met Leu Ala Gln Tyr His	
1 5 10 15	
CAG TTC AAG AGC ATG TAT CCC GAC TGC CTT CTT TTA TTC AGG CTC GGG	96
Gln Phe Lys Ser Met Tyr Pro Asp Cys Leu Leu Leu Phe Arg Leu Gly	
20 25 30	
GAC TTT TAC GAG CTC TTT TAC GAG GAC GCG GTC GTC GGT TCT AAA GAG	144
Asp Phe Tyr Glu Leu Phe Tyr Glu Asp Ala Val Val Gly Ser Lys Glu	
35 40 45	
CTC GGT CTA GTT CTA ACT TCA AGA CCC GCG GGA AAG GGA AGG GAA AGG	192
Leu Gly Leu Val Leu Thr Ser Arg Pro Ala Gly Lys Gly Arg Glu Arg	
50 55 60	
ATT CCC ATG TGC GGT GTT CCC TAC CAT TCT GCA AAC AAC TAT ATA GCA	240
Ile Pro Met Cys Gly Val Pro Tyr His Ser Ala Asn Asn Tyr Ile Ala	
65 70 75 80	
AAG CTC GTT AAT AAG GGA TAC AAG GTA GCA ATA TGC GAG CAG GTT GAG	288
Lys Leu Val Asn Lys Gly Tyr Lys Val Ala Ile Cys Glu Gln Val Glu	
85 90 95	
GAC CCC TCA AAG GCA AAG GGA ATA GTA AAG AGG GAC GTA ATA AGA GTT	336
Asp Pro Ser Lys Ala Lys Gly Ile Val Lys Arg Asp Val Ile Arg Val	
100 105 110	
ATA ACA CCT GGG ACC TTT TTT GAG AGG GAA ACG GGA GGG CTT TGC TCC	384
Ile Thr Pro Gly Thr Phe Phe Glu Arg Glu Thr Gly Gly Leu Cys Ser	
115 120 125	
CTT TAC AGG AAG GGA AAG AGC TAT CTC GTT TCT TAT CTT AAC CTC TCG	432
Leu Tyr Arg Lys Gly Lys Ser Tyr Leu Val Ser Tyr Leu Asn Leu Ser	
130 135 140	
GTA GGT GAG TTC ATA GGT GCA AAG GTA AAG GAG GAA GAG CTC ATA GAC	480
Val Gly Glu Phe Ile Gly Ala Lys Val Lys Glu Glu Glu Leu Ile Asp	
145 150 155 160	
TTC CTC TCA AAG TTC AAC ATA AGG GAG GTT CTT GTA AAG AAG GGA GAA	528
Phe Leu Ser Lys Phe Asn Ile Arg Glu Val Leu Val Lys Lys Gly Glu	
165 170 175	
AAG CTC CCC GAA AAG CTT GAG AAG GTT CTA AAG CTC CAC ATA ACG GAG	576
Lys Leu Pro Glu Lys Leu Glu Lys Val Leu Lys Leu His Ile Thr Glu	
180 185 190	
CTT GAA GAG GAG TTC TTT GAG GAG GGA AAG GAG GAG CTT CTT AAG GAT	624
Leu Glu Glu Glu Phe Phe Glu Glu Gly Lys Glu Glu Leu Leu Lys Asp	
195 200 205	
TAC GGA GTT CCG TCG ATA AAA GCC TTC GGC TTT CAG GAT GAG GAT TTA	672

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Tyr	Gly	Val	Pro	Ser	Ile	Lys	Ala	Phe	Gly	Phe	Gln	Asp	Glu	Asp	Leu	
210						215					220					
TCC	CTT	TCC	CTC	GGG	GCT	GTT	TAC	AGG	TAT	GCA	AAG	GCG	ACA	CAG	AAA	720
Ser	Leu	Ser	Leu	Gly	Ala	Val	Tyr	Arg	Tyr	Ala	Lys	Ala	Thr	Gln	Lys	
225				230						235					240	
TCT	TTT	ACC	CCT	CTC	ATT	CCA	AAG	CCC	AAA	CCT	TAC	GTT	GAC	GAG	GGA	768
Ser	Phe	Thr	Pro	Leu	Ile	Pro	Lys	Pro	Lys	Pro	Tyr	Val	Asp	Glu	Gly	
				245					250					255		
TAC	GTA	AAG	CTT	GAC	CTC	AAG	GCA	GTC	AAA	GGT	CTT	GAG	ATT	ACC	GAA	816
Tyr	Val	Lys	Leu	Asp	Leu	Lys	Ala	Val	Lys	Gly	Leu	Glu	Ile	Thr	Glu	
			260					265					270			
AGC	ATA	GAA	GGA	AGA	AAG	GAT	TTA	TCC	CTG	TTT	AAG	GTC	GTT	GAC	AGA	864
Ser	Ile	Glu	Gly	Arg	Lys	Asp	Leu	Ser	Leu	Phe	Lys	Val	Val	Asp	Arg	
		275					280					285				
ACC	CTC	ACG	GGT	ATG	GGG	AGA	AGG	AGG	CTG	AGG	TTC	AGG	CTT	CTA	AAC	912
Thr	Leu	Thr	Gly	Met	Gly	Arg	Arg	Arg	Leu	Arg	Phe	Arg	Leu	Leu	Asn	
		290				295					300					
CCC	TTC	AGG	AGC	ATA	GAG	AGA	ATA	AGG	AAG	GTT	CAG	GAA	GCA	GTT	GAG	960
Pro	Phe	Arg	Ser	Ile	Glu	Arg	Ile	Arg	Lys	Val	Gln	Glu	Ala	Val	Glu	
305					310					315					320	
GAG	CTA	ATA	AAC	AAG	AGG	GAG	GTT	CTG	AAC	GAG	ATA	AGG	AAA	ACC	CTT	1008
Glu	Leu	Ile	Asn	Lys	Arg	Glu	Val	Leu	Asn	Glu	Ile	Arg	Lys	Thr	Leu	
			325						330					335		
GAG	GGT	ATG	TCC	GAC	CTT	GAG	AGA	CTC	GTA	TCC	AGG	ATA	AGC	TCA	AAC	1056
Glu	Gly	Met	Ser	Asp	Leu	Glu	Arg	Leu	Val	Ser	Arg	Ile	Ser	Ser	Asn	
			340					345				350				
ATG	GCA	AGC	CCA	AGA	GAA	CTT	ATA	CAC	CTC	AAA	AAC	TCC	CTA	AGG	AAG	1104
Met	Ala	Ser	Pro	Arg	Glu	Leu	Ile	His	Leu	Lys	Asn	Ser	Leu	Arg	Lys	
		355					360					365				
GCG	GAG	GAG	CTA	AGG	AAA	ATT	TTA	TCT	TTG	CTT	GAT	TCC	GAA	ATA	TTT	1152
Ala	Glu	Glu	Leu	Arg	Lys	Ile	Leu	Ser	Leu	Leu	Asp	Ser	Glu	Ile	Phe	
	370					375					380					
AAA	GAG	ATA	GAA	GGT	TCT	CTC	CTT	AAC	CTG	AAT	AAA	GTT	GCG	GAC	CTC	1200
Lys	Glu	Ile	Glu	Gly	Ser	Leu	Leu	Asn	Leu	Asn	Lys	Val	Ala	Asp	Leu	
385					390				395					400		
ATT	GAT	AAA	ACG	CTT	GTT	GAC	GAC	CCT	CCC	CTG	CAC	GTA	AAA	GAA	GGG	1248
Ile	Asp	Lys	Thr	Leu	Val	Asp	Asp	Pro	Pro	Leu	His	Val	Lys	Glu	Gly	
			405					410						415		
GGG	CTT	ATA	AAA	CCC	GGT	GTT	AAC	GCA	TAC	CTT	GAT	GAG	CTT	CGC	TTC	1296
Gly	Leu	Ile	Lys	Pro	Gly	Val	Asn	Ala	Tyr	Leu	Asp	Glu	Leu	Arg	Phe	
			420					425					430			
ATA	AGG	GAG	AAT	GCG	GAA	AAG	CTC	CTG	AAG	GAG	TAT	GAA	AAG	AAG	CTG	1344
Ile	Arg	Glu	Asn	Ala	Glu	Lys	Leu	Leu	Lys	Glu	Tyr	Glu	Lys	Lys	Leu	
		435					440					445				
AAA	AAA	GAA	ACG	GGA	ATT	CAG	AGC	TTA	AAG	ATT	GGA	TAC	AAC	AAG	GTT	1392
Lys	Lys	Glu	Thr	Gly	Ile	Gln	Ser	Leu	Lys	Ile	Gly	Tyr	Asn	Lys	Val	
	450					455					460					

ATG GGA TAC TAC ATA GAG GTA ACG AAG GCT AAC GTA AAA TAC GTT CCC Met Gly Tyr Tyr Ile Glu Val Thr Lys Ala Asn Val Lys Tyr Val Pro 465 470 475 480	1440
GAA CAC TTC AGA AGA AGA CAG ACC CTT TCA AAC GCG GAG AGA TAC ACA Glu His Phe Arg Arg Arg Gln Thr Leu Ser Asn Ala Glu Arg Tyr Thr 485 490 495	1488
ACC GAG GAG CTC CAG AGA CTT GAG GAA AAG ATA CTT TCC GCC CAG ACC Thr Glu Glu Leu Gln Arg Leu Glu Lys Ile Leu Ser Ala Gln Thr 500 505 510	1536
CGC ATA AAC GAG CTT GAG TAT GAG CTT TAC AGG GAG CTC AGG GAA GAG Arg Ile Asn Glu Leu Glu Tyr Lys Leu Tyr Arg Glu Leu Arg Glu Glu 515 520 525	1584
GTT GTT AAG GAG CTT GAT AAG GTA GGG AAT AAC GCA ACC CTC ATA GGG Val Val Lys Glu Leu Asp Lys Val Gly Asn Asn Ala Thr Leu Ile Gly 530 535 540	1632
GAG GTG GAC TAC ATC CAG TCC CTC GCC TGG CTT GCC CTT GAG AAG GGA Glu Val Asp Tyr Ile Gln Ser Leu Ala Trp Leu Ala Leu Glu Lys Gly 545 550 555 560	1680
TGG GTA AAG CCG GAA GTT CAC GAG GGA TAT GAG CTG ATA ATA GAG GAG Trp Val Lys Pro Glu Val His Glu Gly Tyr Glu Leu Ile Ile Glu Glu 565 570 575	1728
GGA AAG CAT CCC GTA ATA GAG GAG TTC ACG AAA AAC TAC GTC CCA AAC Gly Lys His Pro Val Ile Glu Glu Phe Thr Lys Asn Tyr Val Pro Asn 580 585 590	1776
GAT ACG AAG CTA ACG GAA GAG GAG TTC ATA CAC GTA ATC ACG GGC CCT Asp Thr Lys Leu Thr Glu Glu Phe Ile His Val Ile Thr Gly Pro 595 600 605	1824
AAC ATG GCG GGA AAG TCG AGC TAC ATA AGA CAG GTG GGC GTC CTC ACG Asn Met Ala Gly Lys Ser Ser Tyr Ile Arg Gln Val Gly Val Leu Thr 610 615 620	1872
CTC CTT GCT CAT ACA GGT AGC TTC CTT CCC GTA AAG AGT GCA AGG ATA Leu Leu Ala His Thr Gly Ser Phe Leu Pro Val Lys Ser Ala Arg Ile 625 630 635 640	1920
CCG CTG GTT GAT GCG ATA TTC ACG AGA ATA GGC TCG GGG GAC GTT CTG Pro Leu Val Asp Ala Ile Phe Thr Arg Ile Gly Ser Gly Asp Val Leu 645 650 655	1968
GCT CTG GGT GTT TCA ACC TTC ATG AAC GAG ATG CTT GAC GTG TCA AAC Ala Leu Gly Val Ser Thr Phe Met Asn Glu Met Leu Asp Val Ser Asn 660 665 670	2016
ATA CTC AAC AAC GCA ACG AAG AGG AGC TTA ATA ATA CTC GAC GAG GTG Ile Leu Asn Asn Ala Thr Lys Arg Ser Leu Ile Ile Leu Asp Glu Val 675 680 685	2064
GGA AGG GGA ACC TCA ACC TAC GAG GGG ATA GCG ATA AGC AAG GCG ATA Gly Arg Gly Thr Ser Thr Tyr Asp Gly Ile Ala Ile Ser Lys Ala Ile 690 695 700	2112
GTG AAA TAC ATA AGC GAG AAG ATA GGG GCG AAA ACG CTA CTC GCA ACC Val Lys Tyr Ile Ser Glu Lys Ile Gly Ala Lys Thr Leu Leu Ala Thr 705 710 715 720	2160

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705	710	715	720	
CAC TAC CTT GAG CTA ACC GAG CTT GAG AGA AAG GTA AAG GGA GTA AAG His Tyr Leu Glu Leu Thr Glu Leu Glu Arg Lys Val Lys Gly Val Lys 725 730 735				2208
AAC TAC CAC ATG GAG GTT GAG GAA ACG GAT GAG GGA ATA AGG TTC TTA Asn Tyr His Met Glu Val Glu Glu Thr Asp Glu Gly Ile Arg Phe Leu 740 745 750				2256
TAC ATA CTG AAG GAG GGA AGG GCG AAG GGA AGC TTC GGC ATA GAC GTC Tyr Ile Leu Lys Glu Gly Arg Ala Lys Gly Ser Phe Gly Ile Asp Val 755 760 765				2304
GCA AAA CTC GCG GGA CTG CCC GAG GAA GTT GTA AGG GAA GCA AAA AAG Ala Lys Leu Ala Gly Leu Pro Glu Glu Val Val Arg Glu Ala Lys Lys 770 775 780				2352
ATA CTG AAG GAG CTT GAA GGG GAA AAA GGA AAG CAG GAA GTT CTC CCC Ile Leu Lys Glu Leu Glu Gly Glu Lys Gly Lys Gln Glu Val Leu Pro 785 790 795 800				2400
TTC CTT GAG GAG ACC TAT AAA AAG TCC GTT GAT GAA GAG AAG CTG AAC Phe Leu Glu Glu Thr Tyr Lys Lys Ser Val Asp Glu Glu Lys Leu Asn 805 810 815				2448
TTT TAC GAA GAG ATA ATA AAG GAG ATA GAG GAG ATA GAT ATA GGG AAC Phe Tyr Glu Glu Ile Ile Lys Glu Ile Glu Glu Ile Asp Ile Gly Asn 820 825 830				2496
ACG ACT CCT GTT AAA GCC CTG CTC ATC CTT GCG GAG TTA AAG GAA AGG Thr Thr Pro Val Lys Ala Leu Leu Ile Leu Ala Glu Leu Lys Glu Arg 835 840 845				2544
ATA AAG AGC TTT ATA AAG AGG TGA Ile Lys Ser Phe Ile Lys Arg 850 855				2568

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 855 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Lys Glu Glu Lys Glu Leu Thr Pro Met Leu Ala Gln Tyr His
1 5 10 15

Gln Phe Lys Ser Met Tyr Pro Asp Cys Leu Leu Leu Phe Arg Leu Gly
20 25 30

Asp Phe Tyr Glu Leu Phe Tyr Glu Asp Ala Val Val Gly Ser Lys Glu
35 40 45

Leu Gly Leu Val Leu Thr Ser Arg Pro Ala Gly Lys Gly Arg Glu Arg
 50 55 60
 Ile Pro Met Cys Gly Val Pro Tyr His Ser Ala Asn Asn Tyr Ile Ala
 65 70 75 80
 Lys Leu Val Asn Lys Gly Tyr Lys Val Ala Ile Cys Glu Gln Val Glu
 85 90 95
 Asp Pro Ser Lys Ala Lys Gly Ile Val Lys Arg Asp Val Ile Arg Val
 100 105 110
 Ile Thr Pro Gly Thr Phe Phe Glu Arg Glu Thr Gly Gly Leu Cys Ser
 115 120 125
 Leu Tyr Arg Lys Gly Lys Ser Tyr Leu Val Ser Tyr Leu Asn Leu Ser
 130 135 140
 Val Gly Glu Phe Ile Gly Ala Lys Val Lys Glu Glu Glu Leu Ile Asp
 145 150 155 160
 Phe Leu Ser Lys Phe Asn Ile Arg Glu Val Leu Val Lys Lys Gly Glu
 165 170 175
 Lys Leu Pro Glu Lys Leu Glu Lys Val Leu Lys Leu His Ile Thr Glu
 180 185 190
 Leu Glu Glu Glu Phe Phe Glu Glu Gly Lys Glu Glu Leu Leu Lys Asp
 195 200 205
 Tyr Gly Val Pro Ser Ile Lys Ala Phe Gly Phe Gln Asp Glu Asp Leu
 210 215 220
 Ser Leu Ser Leu Gly Ala Val Tyr Arg Tyr Ala Lys Ala Thr Gln Lys
 225 230 235 240
 Ser Phe Thr Pro Leu Ile Pro Lys Pro Lys Pro Tyr Val Asp Glu Gly
 245 250 255
 Tyr Val Lys Leu Asp Leu Lys Ala Val Lys Gly Leu Glu Ile Thr Glu
 260 265 270
 Ser Ile Glu Gly Arg Lys Asp Leu Ser Leu Phe Lys Val Val Asp Arg
 275 280 285
 Thr Leu Thr Gly Met Gly Arg Arg Arg Leu Arg Phe Arg Leu Leu Asn
 290 295 300
 Pro Phe Arg Ser Ile Glu Arg Ile Arg Lys Val Gln Glu Ala Val Glu
 305 310 315 320
 Glu Leu Ile Asn Lys Arg Glu Val Leu Asn Glu Ile Arg Lys Thr Leu
 325 330 335
 Glu Gly Met Ser Asp Leu Glu Arg Leu Val Ser Arg Ile Ser Ser Asn
 340 345 350
 Met Ala Ser Pro Arg Glu Leu Ile His Leu Lys Asn Ser Leu Arg Lys
 355 360 365
 Ala Glu Glu Leu Arg Lys Ile Leu Ser Leu Leu Asp Ser Glu Ile Phe
 370 375 380

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Lys Glu Ile Glu Gly Ser Leu Leu Asn Leu Asn Lys Val Ala Asp Leu
 385 390 395 400
 Ile Asp Lys Thr Leu Val Asp Asp Pro Pro Leu His Val Lys Glu Gly
 405 410 415
 Gly Leu Ile Lys Pro Gly Val Asn Ala Tyr Leu Asp Glu Leu Arg Phe
 420 425 430
 Ile Arg Glu Asn Ala Glu Lys Leu Leu Lys Glu Tyr Glu Lys Lys Leu
 435 440 445
 Lys Lys Glu Thr Gly Ile Gln Ser Leu Lys Ile Gly Tyr Asn Lys Val
 450 455 460
 Met Gly Tyr Tyr Ile Glu Val Thr Lys Ala Asn Val Lys Tyr Val Pro
 465 470 475 480
 Glu His Phe Arg Arg Arg Gln Thr Leu Ser Asn Ala Glu Arg Tyr Thr
 485 490 495
 Thr Glu Glu Leu Gln Arg Leu Glu Glu Lys Ile Leu Ser Ala Gln Thr
 500 505 510
 Arg Ile Asn Glu Leu Glu Tyr Glu Leu Tyr Arg Glu Leu Arg Glu Glu
 515 520 525
 Val Val Lys Glu Leu Asp Lys Val Gly Asn Asn Ala Thr Leu Ile Gly
 530 535 540
 Glu Val Asp Tyr Ile Gln Ser Leu Ala Trp Leu Ala Leu Glu Lys Gly
 545 550 555 560
 Trp Val Lys Pro Glu Val His Glu Gly Tyr Glu Leu Ile Ile Glu Glu
 565 570 575
 Gly Lys His Pro Val Ile Glu Glu Phe Thr Lys Asn Tyr Val Pro Asn
 580 585 590
 Asp Thr Lys Leu Thr Glu Glu Glu Phe Ile His Val Ile Thr Gly Pro
 595 600 605
 Asn Met Ala Gly Lys Ser Ser Tyr Ile Arg Gln Val Gly Val Leu Thr
 610 615 620
 Leu Leu Ala His Thr Gly Ser Phe Leu Pro Val Lys Ser Ala Arg Ile
 625 630 635 640
 Pro Leu Val Asp Ala Ile Phe Thr Arg Ile Gly Ser Gly Asp Val Leu
 645 650 655
 Ala Leu Gly Val Ser Thr Phe Met Asn Glu Met Leu Asp Val Ser Asn
 660 665 670
 Ile Leu Asn Asn Ala Thr Lys Arg Ser Leu Ile Ile Leu Asp Glu Val
 675 680 685
 Gly Arg Gly Thr Ser Thr Tyr Asp Gly Ile Ala Ile Ser Lys Ala Ile
 690 695 700
 Val Lys Tyr Ile Ser Glu Lys Ile Gly Ala Lys Thr Leu Leu Ala Thr
 705 710 715 720

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His Tyr Leu Glu Leu Thr Glu Leu Glu Arg Lys Val Lys Gly Val Lys
 725 730 735
 Asn Tyr His Met Glu Val Glu Glu Thr Asp Glu Gly Ile Arg Phe Leu
 740 745 750
 Tyr Ile Leu Lys Glu Gly Arg Ala Lys Gly Ser Phe Gly Ile Asp Val
 755 760 765
 Ala Lys Leu Ala Gly Leu Pro Glu Glu Val Val Arg Glu Ala Lys Lys
 770 775 780
 Ile Leu Lys Glu Leu Glu Gly Glu Lys Gly Lys Gln Glu Val Leu Pro
 785 790 795 800
 Phe Leu Glu Glu Thr Tyr Lys Lys Ser Val Asp Glu Glu Lys Leu Asn
 805 810 815
 Phe Tyr Glu Glu Ile Ile Lys Glu Ile Glu Glu Ile Asp Ile Gly Asn
 820 825 830
 Thr Thr Pro Val Lys Ala Leu Leu Ile Leu Ala Glu Leu Lys Glu Arg
 835 840 845
 Ile Lys Ser Phe Ile Lys Arg
 850 855

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 853 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Ser Ala Ile Glu Asn Phe Asp Ala His Thr Pro Met Met Gln Gln
 1 5 10 15
 Tyr Leu Arg Leu Lys Ala Gln His Pro Glu Ile Leu Leu Phe Tyr Arg
 20 25 30
 Met Gly Asp Phe Tyr Glu Leu Phe Tyr Asp Asp Ala Lys Arg Ala Ser
 35 40 45
 Gln Leu Leu Asp Ile Ser Leu Thr Lys Arg Gly Ala Ser Ala Gly Glu
 50 55 60
 Pro Ile Pro Met Ala Gly Ile Pro Tyr His Ala Val Glu Asn Tyr Leu
 65 70 75 80
 Ala Lys Leu Val Asn Gln Gly Glu Ser Val Ala Ile Cys Glu Gln Ile
 85 90 95
 Gly Asp Pro Ala Thr Ser Lys Gly Pro Val Glu Arg Lys Val Val Arg

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100					105					110					
Ile	Val	Thr	Pro	Gly	Thr	Ile	Ser	Asp	Glu	Ala	Leu	Leu	Gln	Glu	Arg
		115					120					125			
Gln	Asp	Asn	Leu	Leu	Ala	Ala	Ile	Trp	Gln	Asp	Ser	Lys	Gly	Phe	Gly
	130					135					140				
Tyr	Ala	Thr	Leu	Asp	Ile	Ser	Ser	Gly	Arg	Phe	Arg	Leu	Ser	Glu	Pro
	145					150					155				160
Ala	Asp	Arg	Glu	Thr	Met	Ala	Ala	Glu	Leu	Gln	Arg	Thr	Asn	Pro	Ala
				165					170					175	
Glu	Leu	Leu	Tyr	Ala	Glu	Asp	Phe	Ala	Glu	Met	Ser	Leu	Ile	Glu	Gly
			180					185					190		
Arg	Arg	Gly	Leu	Arg	Arg	Arg	Pro	Leu	Trp	Glu	Phe	Glu	Ile	Asp	Thr
		195					200					205			
Ala	Arg	Gln	Gln	Leu	Asn	Leu	Gln	Phe	Gly	Thr	Arg	Asp	Leu	Val	Gly
	210					215					220				
Phe	Gly	Val	Glu	Asn	Ala	Pro	Arg	Gly	Leu	Cys	Ala	Ala	Gly	Cys	Leu
	225					230					235				240
Leu	Gln	Tyr	Ala	Lys	Asp	Thr	Gln	Arg	Thr	Thr	Leu	Pro	His	Ile	Arg
				245					250					255	
Ser	Ile	Thr	Met	Glu	Arg	Glu	Gln	Asp	Ser	Ile	Ile	Met	Asp	Ala	Ala
			260					265					270		
Thr	Arg	Arg	Asn	Leu	Glu	Ile	Thr	Gln	Asn	Leu	Ala	Gly	Gly	Ala	Glu
		275					280					285			
Asn	Thr	Leu	Ala	Ser	Val	Leu	Asp	Cys	Thr	Val	Thr	Pro	Met	Gly	Ser
	290					295					300				
Arg	Met	Leu	Lys	Arg	Trp	Leu	His	Met	Pro	Val	Arg	Asp	Thr	Arg	Val
	305					310					315				320
Leu	Leu	Glu	Arg	Gln	Gln	Thr	Ile	Gly	Ala	Leu	Gln	Asp	Phe	Thr	Ala
				325					330					335	
Gly	Leu	Gln	Pro	Val	Leu	Arg	Gln	Val	Gly	Asp	Leu	Glu	Arg	Ile	Leu
			340					345					350		
Ala	Arg	Leu	Ala	Leu	Arg	Thr	Ala	Arg	Pro	Arg	Asp	Leu	Ala	Arg	Met
			355				360					365			
Arg	His	Ala	Phe	Gln	Gln	Leu	Pro	Glu	Leu	Arg	Ala	Gln	Leu	Glu	Thr
	370					375					380				
Val	Asp	Ser	Ala	Pro	Val	Gln	Ala	Leu	Arg	Glu	Lys	Met	Gly	Glu	Phe
	385					390					395				400
Ala	Glu	Leu	Arg	Asp	Leu	Leu	Glu	Arg	Ala	Ile	Ile	Asp	Thr	Pro	Pro
				405				410						415	
Val	Leu	Val	Arg	Asp	Gly	Gly	Val	Ile	Ala	Ser	Gly	Tyr	Asn	Glu	Glu
				420				425					430		

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Leu Asp Glu Trp Arg Ala Leu Ala Asp Gly Ala Thr Asp Tyr Leu Glu
 435 440 445
 Arg Leu Glu Val Arg Glu Arg Glu Arg Thr Gly Leu Asp Thr Leu Lys
 450 455 460
 Val Gly Phe Asn Ala Val His Gly Tyr Tyr Ile Gln Ile Ser Arg Gly
 465 470 475 480
 Gln Ser His Leu Ala Pro Ile Asn Tyr Met Arg Arg Gln Thr Leu Lys
 485 490 495
 Asn Ala Glu Arg Tyr Ile Ile Pro Glu Leu Lys Glu Tyr Glu Asp Lys
 500 505 510
 Val Leu Thr Ser Lys Gly Lys Ala Leu Ala Leu Glu Lys Gln Leu Tyr
 515 520 525
 Glu Glu Leu Phe Asp Leu Leu Leu Pro His Leu Glu Ala Leu Gln Gln
 530 535 540
 Ser Ala Ser Ala Leu Ala Glu Leu Asp Val Leu Val Asn Leu Ala Glu
 545 550 555 560
 Arg Ala Tyr Thr Leu Asn Tyr Thr Cys Pro Thr Phe Ile Asp Lys Pro
 565 570 575
 Gly Ile Arg Ile Thr Glu Gly Arg His Pro Val Val Glu Gln Val Leu
 580 585 590
 Asn Glu Pro Phe Ile Ala Asn Pro Leu Asn Leu Ser Pro Gln Arg Arg
 595 600 605
 Met Leu Ile Ile Thr Gly Pro Asn Met Gly Gly Lys Ser Thr Tyr Met
 610 615 620
 Arg Gln Thr Ala Leu Ile Ala Leu Met Ala Tyr Ile Gly Ser Tyr Val
 625 630 635 640
 Pro Ala Gln Lys Val Glu Ile Gly Pro Ile Asp Arg Ile Phe Thr Arg
 645 650 655
 Val Gly Ala Ala Asp Asp Leu Ala Ser Gly Arg Ser Thr Phe Met Val
 660 665 670
 Glu Met Thr Glu Thr Ala Asn Ile Leu His Asn Ala Thr Glu Tyr Ser
 675 680 685
 Leu Val Leu Met Asp Glu Ile Gly Arg Gly Thr Ser Thr Tyr Asp Gly
 690 695 700
 Leu Ser Leu Ala Trp Ala Cys Ala Glu Asn Leu Ala Asn Lys Ile Lys
 705 710 715 720
 Ala Leu Thr Leu Phe Ala Thr His Tyr Phe Glu Leu Thr Gln Leu Pro
 725 730 735
 Glu Lys Met Glu Gly Val Ala Asn Val His Leu Asp Ala Leu Glu His
 740 745 750
 Gly Asp Thr Ile Ala Phe Met His Ser Val Gln Asp Gly Ala Ala Ser
 755 760 765

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Lys Ser Tyr Gly Leu Ala Val Ala Ala Leu Ala Gly Val Pro Lys Glu
 770 775 780
 Val Ile Lys Arg Ala Arg Gln Lys Leu Arg Glu Leu Glu Ser Ile Ser
 785 790 795 800
 Pro Asn Ala Ala Ala Thr Gln Val Asp Gly Thr Gln Met Ser Leu Leu
 805 810 815
 Ser Val Pro Glu Glu Thr Ser Pro Ala Val Glu Ala Leu Glu Asn Leu
 820 825 830
 Asp Pro Asp Ser Leu Thr Pro Arg Gln Ala Leu Glu Trp Ile Tyr Arg
 835 840 845
 Leu Lys Ser Leu Val
 850

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2382 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
- (A) NAME/KEY: CDS
 - (B) LOCATION: 1..2379

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTG AAG GTA ACT CCC CTC ATG GAA CAG TAC CTG AGA ATA AAA GAA CAG	48
Val Lys Val Thr Pro Leu Met Glu Gln Tyr Leu Arg Ile Lys Glu Gln	
1 5 10 15	
TAC AAA GAT TCC ATT CTG CTG TTT CGA CTG GGA GAT TTT TAC GAG GCG	96
Tyr Lys Asp Ser Ile Leu Leu Phe Arg Leu Gly Asp Phe Tyr Glu Ala	
20 25 30	
TTT TTC GAA GAC GCA AAG ATC GTT TCG AAG GTT CTG AAC ATA GTT CTC	144
Phe Phe Glu Asp Ala Lys Ile Val Ser Lys Val Leu Asn Ile Val Leu	
35 40 45	
ACA AGA AGG CAG GAC GCT CCC ATG GCG GGC ATC CCG TAC CAC GCG CTG	192
Thr Arg Arg Gln Asp Ala Pro Met Ala Gly Ile Pro Tyr His Ala Leu	
50 55 60	
AAC ACC TAC CTG AAA AAG CTC GTC GAA GCG GGC TAC AAG GTG GCA ATC	240
Asn Thr Tyr Leu Lys Lys Leu Val Glu Ala Gly Tyr Lys Val Ala Ile	
65 70 75 80	
TGC GAT CAA ATG GAA GAA CCT TCG AAG TCG AAG AAA TTG ATC AGA AGG	288
Cys Asp Gln Met Glu Glu Pro Ser Lys Ser Lys Lys Leu Ile Arg Arg	
85 90 95	

GAA GTC ACG CGC GTT GTC ACT CCC GGC TCC ATC GTA GAG GAT GAG TTT	336
Glu Val Thr Arg Val Val Thr Pro Gly Ser Ile Val Glu Asp Glu Phe	
100 105 110	
CTC AGC GAA ACG AAC AAC TAC ATG GCC GTT GTC TCA GAA GAG AAA GGA	384
Leu Ser Glu Thr Asn Asn Tyr Met Ala Val Val Ser Glu Glu Lys Gly	
115 120 125	
CGG TAC TGT ACG GTT TTC TGT GAT GTC TCG ACA GGT GAG GTC CTG GTT	432
Arg Tyr Cys Thr Val Phe Cys Asp Val Ser Thr Gly Glu Val Leu Val	
130 135 140	
CAT GAA AGT TCA GAC GAA CAG GAA ACT TTG GAC CTG CTG AAG AAT TAC	480
His Glu Ser Ser Asp Glu Gln Glu Thr Leu Asp Leu Leu Lys Asn Tyr	
145 150 155 160	
TCC ATT TCC CAG ATC ATC TGT CCA GAG CAC CTG AAA TCT TCT TTG AAG	528
Ser Ile Ser Gln Ile Ile Cys Pro Glu His Leu Lys Ser Ser Trp Lys	
165 170 175	
GAA CGC TTT CCA GGT GTT TAC ACA GAA ACC ATA AGC GAG TGG TAT TTC	576
Glu Arg Phe Pro Gly Val Tyr Thr Glu Thr Ile Ser Glu Trp Tyr Phe	
180 185 190	
TCA GAT CTG GAA GAA GTG GAA AAA GCC TAC AAT CTG AAA GAC ATT CAT	624
Ser Asp Leu Glu Glu Val Glu Lys Ala Tyr Asn Leu Lys Asp Ile His	
195 200 205	
CAT TTC GAG CTT TCG CCC CTT GCG CTG AAA GCC CTT GCG GCG CTG ATA	672
His Phe Glu Leu Ser Pro Leu Ala Leu Lys Ala Leu Ala Ala Leu Ile	
210 215 220	
AAG TAT GTC AAG TAC ACG ATG ATC GGG GAA GAT CTG AAT CTG AAA CCC	720
Lys Tyr Val Lys Tyr Thr Met Ile Gly Glu Asp Leu Asn Leu Lys Pro	
225 230 235 240	
CCT CTT CTC ATC TCC CAG AGA GAC TAC ATG ATA CTC GAT TCC GCA ACG	768
Pro Leu Leu Ile Ser Gln Arg Asp Tyr Met Ile Leu Asp Ser Ala Thr	
245 250 255	
GTG GAA AAT CTT TCT TGG ATT CCC GGT GAC AGG GGA AAG AAT CTT TTC	816
Val Glu Asn Leu Ser Trp Ile Pro Gly Asp Arg Gly Lys Asn Leu Phe	
260 265 270	
GAT GTG CTG AAC AAC ACG GAA ACT CCT ATG GGG GCT CGT CTT GGG AAA	864
Asp Val Leu Asn Asn Thr Glu Thr Pro Met Gly Ala Arg Leu Gly Lys	
275 280 285	
AAG TGG ATT CTC CAC CCT CTG GTC GAC AGA AAA CAG ATC GAA GAA AGG	912
Lys Trp Ile Leu His Pro Leu Val Asp Arg Lys Gln Ile Glu Glu Arg	
290 295 300	
CTC AAG GCT GTG GAA AGA CTG GTG AAC GAC AGG GTG AGC CTG GAG GAG	960
Leu Lys Ala Val Glu Arg Leu Val Asn Asp Arg Val Ser Leu Glu Glu	
305 310 315 320	
ATG AGG AAC CTT CTT TCG AAC GTG AGG GAT GTG GAG CGG ATC GTT TCG	1008
Met Arg Asn Leu Leu Ser Asn Val Arg Asp Val Glu Arg Ile Val Ser	
325 330 335	
CGG GTG GAG TAC AAC AGA TCC GTT CCC AGG GAC TTA GTG GCA CTC AGA	1056
Arg Val Glu Tyr Asn Arg Ser Val Pro Arg Asp Leu Val Ala Leu Arg	

340						345						350						
GAG Glu	ACA Thr	CTG Leu 355	GAG Glu	ATC Ile	ATC Ile	CCG Pro	AAA Lys 360	CTG Leu	AAC Asn	GAA Glu	GTT Val	CTT Leu 365	TCA Ser	ACC Thr	TTC Phe	1104		
GGT Gly	GTG Val 370	TTC Phe	AAG Lys	AAA Lys	CTC Leu	GCT Ala 375	TTC Phe	CCG Pro	GAA Glu	GGA Gly	CTG Leu 380	GTT Val	GAT Asp	CTG Leu	CTT Leu	1152		
CGA Arg 385	AAA Lys	GCC Ala	ATT Ile	GAA Glu	GAT Asp 390	GAT Asp	CCG Pro	GTG Val	GGA Gly	AGC Ser 395	CCC Pro	GGC Gly	GAG Glu	GGA Gly	AAA Lys 400	1200		
GTT Val	ATA Ile	AAG Lys	AGA Arg	GGA Gly 405	TTC Phe	TCA Ser	TCT Ser	GAA Glu	CTC Leu 410	GAC Asp	GAA Glu	TAC Tyr	AGG Arg	GAT Asp 415	CTT Leu	1248		
CTG Leu	GAA Glu	CAT His	GCC Ala 420	GAA Glu	GAG Glu	AGG Arg	CTC Leu	AAA Lys 425	GAG Glu	TTC Phe	GAG Glu	GAG Glu	AAG Lys 430	GAG Glu	AGA Arg	1296		
GAA Glu	AGA Arg	ACA Thr 435	GGC Gly	ATC Ile	CAA Gln	AAA Lys	CTG Leu 440	CGG Arg	GTT Val	GGA Gly	TAC Tyr	AAC Asn 445	CAG Gln	GTT Val	TTT Phe	1344		
GGT Gly	TAC Tyr 450	TAC Tyr	ATA Ile	GAG Glu	GTG Val	ACG Thr 455	AAG Lys	GCG Ala	AAT Asn	CTG Leu	GAT Asp 460	AAG Lys	ATT Ile	CCC Pro	GAC Asp	1392		
GAT Asp 465	TAC Tyr	GAA Glu	AGA Arg	AAA Lys	CAA Gln 470	ACA Thr	CTC Leu	GTC Val	AAT Asn	TCT Ser 475	GAA Glu	AGA Arg	TTC Phe	ATC Ile	ACA Thr 480	1440		
CCC Pro	GAA Glu	TTG Leu	AAG Lys	GAG Glu 485	TTC Phe	GAG Glu	ACA Thr	AAG Lys	ATA Ile 490	ATG Met	GCC Ala	GCT Ala	AAA Lys	GAG Glu 495	AGA Arg	1488		
ATA Ile	GAA Glu	GAA Glu	CTG Leu 500	GAA Glu	AAG Lys	GAA Glu	CTC Leu	TTC Phe 505	ACA Thr	AGC Ser	GTG Val	TGC Cys	GAA Glu 510	GAG Glu	GTG Val	1536		
AAA Lys	AAG Lys	CAC His 515	AAA Lys	GAA Glu	GTT Val	CTC Leu	CTT Leu 520	GAG Glu	ATC Ile	TCG Ser	GAG Glu	GAT Asp 525	CTG Leu	GCA Ala	AAG Lys	1584		
ATA Ile	GAT Asp 530	GCG Ala	CTT Leu	TCG Ser	ACG Thr	TTA Leu 535	GCA Ala	TAC Tyr	GAC Asp	GCT Ala	ATT Ile 540	ATG Met	TAC Tyr	AAC Asn	TAC Tyr	1632		
ACA Thr 545	AAA Lys	CCC Pro	GTC Val	TTT Phe	TCA Ser 550	GAA Glu	GAC Asp	AGA Arg	CTG Leu	GAG Glu 555	ATC Ile	AAA Lys	GGT Gly	GGA Gly	AGA Arg 560	1680		
CAC His	CCG Pro	GTC Val	GTT Val	GAA Glu 565	AGG Arg	TTC Phe	ACA Thr	CAG Gln	AAT Asn 570	TTT Phe	GTT Val	GAA Glu	AAC Asn	GAT Asp 575	ATT Ile	1728		
TAC Tyr	ATG Met	GAC Asp	AAC Asn 580	GAG Glu	AAG Lys	AGA Arg	TTT Phe 585	GTG Val	GTA Val	ATA Ile	ACG Thr	GGT Gly	CCC Pro 590	AAC Asn	ATG Met	1776		

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AGC	GGG	AAG	TCC	ACT	TTC	ATC	AGA	CAG	GTG	GGT	CTC	ATA	TCC	CTC	ATG	1824
Ser	Gly	Lys	Ser	Thr	Phe	Ile	Arg	Gln	Val	Gly	Leu	Ile	Ser	Leu	Met	
		595					600					605				
GCG	CAG	ATA	GGA	TCG	TTT	GTG	CCG	GCG	CAG	AAG	GCG	ATT	CTT	CCA	GTG	1872
Ala	Gln	Ile	Gly	Ser	Phe	Val	Pro	Ala	Gln	Lys	Ala	Ile	Leu	Pro	Val	
	610					615					620					
TTC	GAC	AGG	ATT	TTC	ACG	CGA	ATG	GGT	GCC	AGA	GAC	GAT	CTC	GCT	GGT	1920
Phe	Asp	Arg	Ile	Phe	Thr	Arg	Met	Gly	Ala	Arg	Asp	Asp	Leu	Ala	Gly	
625					630					635					640	
GGT	AGA	AGT	ACG	TTC	CTT	GTC	GAG	ATG	AAC	GAG	ATG	GCG	CTC	ATC	CTT	1968
Gly	Arg	Ser	Thr	Phe	Leu	Val	Glu	Met	Asn	Glu	Met	Ala	Leu	Ile	Leu	
			645						650					655		
CTG	AAA	TCA	ACA	AAT	AAG	AGT	CTG	GTT	CTC	CTG	GAC	GAG	GTG	GGA	AGA	2016
Leu	Lys	Ser	Thr	Asn	Lys	Ser	Leu	Val	Leu	Leu	Asp	Glu	Val	Gly	Arg	
			660					665					670			
GGT	ACA	AGC	ACC	CAG	GAC	GGC	GTC	AGC	ATA	GCC	TGG	GCA	ATC	TCA	GAG	2064
Gly	Thr	Ser	Thr	Gln	Asp	Gly	Val	Ser	Ile	Ala	Trp	Ala	Ile	Ser	Glu	
		675					680					685				
GAA	CTC	ATA	AAG	AGA	GGA	TGT	AAG	GTG	CTG	TTT	GCC	ACT	CAT	TTC	ACG	2112
Glu	Leu	Ile	Lys	Arg	Gly	Cys	Lys	Val	Leu	Phe	Ala	Thr	His	Phe	Thr	
	690					695					700					
GAA	CTC	ACG	GAA	CTC	GAA	AAA	CAC	TTT	CCG	CAG	GTT	CAG	AAC	AAA	ACC	2160
Glu	Leu	Thr	Glu	Leu	Glu	Lys	His	Phe	Pro	Gln	Val	Gln	Asn	Lys	Thr	
705					710					715					720	
ATT	CTG	GTA	AAA	GAA	GAA	GGC	AAA	AAC	GTG	ATA	TTC	ACC	CAC	AAG	GTG	2208
Ile	Leu	Val	Lys	Glu	Glu	Gly	Lys	Asn	Val	Ile	Phe	Thr	His	Lys	Val	
			725						730					735		
GTG	GAC	GGT	GTG	GCA	GAC	AGA	AGT	TAC	GGA	ATA	GAG	GTC	GCA	AAG	ATA	2256
Val	Asp	Gly	Val	Ala	Asp	Arg	Ser	Tyr	Gly	Ile	Glu	Val	Ala	Lys	Ile	
			740					745					750			
GCG	GGT	ATT	CCT	GAC	AGG	GTT	ATA	AAC	AGA	GCC	TAT	GAA	ATT	CTG	GAG	2304
Ala	Gly	Ile	Pro	Asp	Arg	Val	Ile	Asn	Arg	Ala	Tyr	Glu	Ile	Leu	Glu	
		755					760					765				
AGG	AAT	TTC	AAA	AAC	AAC	ACG	AAG	AAA	AAC	GGA	AAA	TCG	AAC	AGA	TTC	2352
Arg	Asn	Phe	Lys	Asn	Asn	Thr	Lys	Lys	Asn	Gly	Lys	Ser	Asn	Arg	Phe	
	770					775					780					
AGT	CAG	CAA	ATT	CCT	CTC	TTT	CCT	GTT	TGA							2382
Ser	Gln	Gln	Ile	Pro	Leu	Phe	Pro	Val								
785						790										

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 793 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Val Lys Val Thr Pro Leu Met Glu Gln Tyr Leu Arg Ile Lys Glu Gln
 1 5 10 15
 Tyr Lys Asp Ser Ile Leu Leu Phe Arg Leu Gly Asp Phe Tyr Glu Ala
 20 25 30
 Phe Phe Glu Asp Ala Lys Ile Val Ser Lys Val Leu Asn Ile Val Leu
 35 40 45
 Thr Arg Arg Gln Asp Ala Pro Met Ala Gly Ile Pro Tyr His Ala Leu
 50 55 60
 Asn Thr Tyr Leu Lys Lys Leu Val Glu Ala Gly Tyr Lys Val Ala Ile
 65 70 75 80
 Cys Asp Gln Met Glu Glu Pro Ser Lys Ser Lys Lys Leu Ile Arg Arg
 85 90 95
 Glu Val Thr Arg Val Val Thr Pro Gly Ser Ile Val Glu Asp Glu Phe
 100 105 110
 Leu Ser Glu Thr Asn Asn Tyr Met Ala Val Val Ser Glu Glu Lys Gly
 115 120 125
 Arg Tyr Cys Thr Val Phe Cys Asp Val Ser Thr Gly Glu Val Leu Val
 130 135 140
 His Glu Ser Ser Asp Glu Gln Glu Thr Leu Asp Leu Leu Lys Asn Tyr
 145 150 155 160
 Ser Ile Ser Gln Ile Ile Cys Pro Glu His Leu Lys Ser Ser Leu Lys
 165 170 175
 Glu Arg Phe Pro Gly Val Tyr Thr Glu Thr Ile Ser Glu Trp Tyr Phe
 180 185 190
 Ser Asp Leu Glu Glu Val Glu Lys Ala Tyr Asn Leu Lys Asp Ile His
 195 200 205
 His Phe Glu Leu Ser Pro Leu Ala Leu Lys Ala Leu Ala Ala Leu Ile
 210 215 220
 Lys Tyr Val Lys Tyr Thr Met Ile Gly Glu Asp Leu Asn Leu Lys Pro
 225 230 235 240
 Pro Leu Leu Ile Ser Gln Arg Asp Tyr Met Ile Leu Asp Ser Ala Thr
 245 250 255
 Val Glu Asn Leu Ser Trp Ile Pro Gly Asp Arg Gly Lys Asn Leu Phe
 260 265 270
 Asp Val Leu Asn Asn Thr Glu Thr Pro Met Gly Ala Arg Leu Gly Lys
 275 280 285
 Lys Trp Ile Leu His Pro Leu Val Asp Arg Lys Gln Ile Glu Glu Arg
 290 295 300
 Leu Lys Ala Val Glu Arg Leu Val Asn Asp Arg Val Ser Leu Glu Glu

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305		310		315		320
Met Arg Asn Leu Leu Ser Asn Val Arg Asp Val Glu Arg Ile Val Ser						
		325		330		335
Arg Val Glu Tyr Asn Arg Ser Val Pro Arg Asp Leu Val Ala Leu Arg						
		340		345		350
Glu Thr Leu Glu Ile Ile Pro Lys Leu Asn Glu Val Leu Ser Thr Phe						
		355		360		365
Gly Val Phe Lys Lys Leu Ala Phe Pro Glu Gly Leu Val Asp Leu Leu						
		370		375		380
Arg Lys Ala Ile Glu Asp Asp Pro Val Gly Ser Pro Gly Glu Gly Lys						
		385		390		395
Val Ile Lys Arg Gly Phe Ser Ser Glu Leu Asp Glu Tyr Arg Asp Leu						
		405		410		415
Leu Glu His Ala Glu Glu Arg Leu Lys Glu Phe Glu Glu Lys Glu Arg						
		420		425		430
Glu Arg Thr Gly Ile Gln Lys Leu Arg Val Gly Tyr Asn Gln Val Phe						
		435		440		445
Gly Tyr Tyr Ile Glu Val Thr Lys Ala Asn Leu Asp Lys Ile Pro Asp						
		450		455		460
Asp Tyr Glu Arg Lys Gln Thr Leu Val Asn Ser Glu Arg Phe Ile Thr						
		465		470		475
Pro Glu Leu Lys Glu Phe Glu Thr Lys Ile Met Ala Ala Lys Glu Arg						
		485		490		495
Ile Glu Glu Leu Glu Lys Glu Leu Phe Thr Ser Val Cys Glu Glu Val						
		500		505		510
Lys Lys His Lys Glu Val Leu Leu Glu Ile Ser Glu Asp Leu Ala Lys						
		515		520		525
Ile Asp Ala Leu Ser Thr Leu Ala Tyr Asp Ala Ile Met Tyr Asn Tyr						
		530		535		540
Thr Lys Pro Val Phe Ser Glu Asp Arg Leu Glu Ile Lys Gly Gly Arg						
		545		550		555
His Pro Val Val Glu Arg Phe Thr Gln Asn Phe Val Glu Asn Asp Ile						
		565		570		575
Tyr Met Asp Asn Glu Lys Arg Phe Val Val Ile Thr Gly Pro Asn Met						
		580		585		590
Ser Gly Lys Ser Thr Phe Ile Arg Gln Val Gly Leu Ile Ser Leu Met						
		595		600		605
Ala Gln Ile Gly Ser Phe Val Pro Ala Gln Lys Ala Ile Leu Pro Val						
		610		615		620
Phe Asp Arg Ile Phe Thr Arg Met Gly Ala Arg Asp Asp Leu Ala Gly						
		625		630		635
						640

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Gly Arg Ser Thr Phe Leu Val Glu Met Asn Glu Met Ala Leu Ile Leu
 645 650 655
 Leu Lys Ser Thr Asn Lys Ser Leu Val Leu Leu Asp Glu Val Gly Arg
 660 665 670
 Gly Thr Ser Thr Gln Asp Gly Val Ser Ile Ala Trp Ala Ile Ser Glu
 675 680 685
 Glu Leu Ile Lys Arg Gly Cys Lys Val Leu Phe Ala Thr His Phe Thr
 690 695 700
 Glu Leu Thr Glu Leu Glu Lys His Phe Pro Gln Val Gln Asn Lys Thr
 705 710 715 720
 Ile Leu Val Lys Glu Glu Gly Lys Asn Val Ile Phe Thr His Lys Val
 725 730 735
 Val Asp Gly Val Ala Asp Arg Ser Tyr Gly Ile Glu Val Ala Lys Ile
 740 745 750
 Ala Gly Ile Pro Asp Arg Val Ile Asn Arg Ala Tyr Glu Ile Leu Glu
 755 760 765
 Arg Asn Phe Lys Asn Asn Thr Lys Lys Asn Gly Lys Ser Asn Arg Phe
 770 775 780
 Ser Gln Gln Ile Pro Leu Phe Pro Val
 785 790

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 102 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAGTCCACCT TCCTCCGCCG GACCGCCCTC ATCGCCCTCC TCGCCCAGAT CGGGAGCTTC 60
 GCGCCCGCCG AGGGGCTGCT GCTTCCCTC TTTGACGGGA TC 102

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 102 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAGTCCACCT TTCTGCGCCA GACGGCCCTC ATCGCCCTCC TGGCCCAGGT GGGGAGCTTC 60
 GTGCCCCGCG AGGAGGCCCA TCTTCCCCTC TTTGACGGCA TC 102

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 34 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Ser Thr Phe Leu Arg Gln Thr Ala Leu Ile Ala Leu Leu Ala Gln
 1 5 10 15
 Val Gly Ser Phe Val Pro Ala Glu Glu Ala His Leu Pro Leu Phe Asp
 20 25 30
 Gly Ile

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 34 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Ser Thr Phe Leu Arg Arg Thr Ala Leu Ile Ala Leu Leu Ala Gln
 1 5 10 15
 Ile Gly Ser Phe Ala Pro Ala Glu Gly Leu Leu Leu Pro Leu Phe Asp
 20 25 30
 Gly Ile

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGGATCCTC

10

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGGACCCCTC

9

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 11 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGGGATCCCT C

11

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 13 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGGGATCCCC CTC

13

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(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TACGCCAGCT GGC GAAAGGG

20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AATGCAGCTG GCACGACAGG

20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GACTCTAGAG GATCCATGT

19

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PCR primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AUGAUGAUGA UGAUCGCACA TTTCCCCGAA AAGTG

35

- (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PCR primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AUCAUCAUCA UCAUGCGCGG AACCCCTATT TGT

33

- (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCGGAATTCC SAACATGGGS GGNA

25

- (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "Primer"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GCGAGATCTA AGTAGTGS GT NGCRAA

26

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCGAGATCTC ACCTGTCTTA TGTAGCTCGA

30

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCGAGATCTC ATCTCGACAA GGAACGTACT

30

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GCGGAATTCA TGGGGGAYTT YTAYGA

26

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCGGAATTCG GGAAAGGATT CCCATGTTTCG

30

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GCGAGATCTC CTTTCCAGCG GGTCTTGAAG

30

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GCGGAATTCC GGGCATCCCG TACCACTCGC

30

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GCGAGATCTG GAGCGTCCCT GCCCTTCTTG

30

- (2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCGGAATTCT CAACCTTCAT GAACGAGATG

30

- (2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GCGAGATCTC GAGCCTATTC TCATGAATAT

30

- (2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid

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(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GCGGAATTCG AGGTGGGAAG AGGTACAAGC

30

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GCGAGATCTC ATCTCGACAA GGAACGTACT

30

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GCGAAGCTTA TGAAGGTAAC TCCCCTCATG

30

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

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GCGGGATCCA CGCATCGATA CTGGTTAAAA

30

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GCGCCATGGG AAAAGAGGAG AAAGAGCTCA

30

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GCGAGATCTG ATACTCCAGA GGTATTACAA

30

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

AATGCAGCTG GCACGACAGG

20

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(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GGTACCCGGG GATCCTCTAG

20

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TACCCGGGGA TCCTCTAGAG

20

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1380 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 61..1338

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GAATTCCTAA GGTCTCAAG GGCTGTTCTT TTCTCTTTT CCTTCCTAAT TTAATACCTC

60

ATG TTT GTC AAA ATC CTG CCC CCA GAG GTA AGG AGA AAG ATT GCA GCG
Met Phe Val Lys Ile Leu Pro Pro Glu Val Arg Arg Lys Ile Ala Ala
1 5 10 15

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GGA	GAG	GTT	ATA	GAC	GCT	CCC	GTT	GAC	GTT	GTA	AAA	GAG	CTT	ATA	GAG	156
Gly	Glu	Val	Ile	Asp	Ala	Pro	Val	Asp	Val	Val	Lys	Glu	Leu	Ile	Glu	
			20					25					30			
AAC	TCC	CTT	GAC	GCT	AAG	GCA	ACG	AGG	ATT	GAG	ATT	GAG	GTC	GTA	AAA	204
Asn	Ser	Leu	Asp	Ala	Lys	Ala	Thr	Arg	Ile	Glu	Ile	Glu	Val	Val	Lys	
		35					40					45				
GGG	GGG	AAA	AGA	CTT	ATC	AGA	GTT	AAG	GAT	AAC	GGG	ATA	GGC	ATT	CAT	252
Gly	Gly	Lys	Arg	Leu	Ile	Arg	Val	Lys	Asp	Asn	Gly	Ile	Gly	Ile	His	
	50					55					60					
CCC	GAG	GAT	ATA	GAA	AAG	GTC	GTT	TTA	TCG	GGA	GCT	ACG	AGC	AAG	ATA	300
Pro	Glu	Asp	Ile	Glu	Lys	Val	Val	Leu	Ser	Gly	Ala	Thr	Ser	Lys	Ile	
	65				70					75					80	
GAG	AAG	GAA	ACG	GAC	CTC	CTC	AAT	GTG	GAA	ACC	TAC	GGA	TTC	AGG	GGG	348
Glu	Lys	Glu	Thr	Asp	Leu	Leu	Asn	Val	Glu	Thr	Tyr	Gly	Phe	Arg	Gly	
				85					90					95		
GAA	GCC	CTG	TAT	TCC	ATC	TCA	AGC	GTA	AGC	AAG	TTC	AGG	CTA	AGG	TCA	396
Glu	Ala	Leu	Tyr	Ser	Ile	Ser	Ser	Val	Ser	Lys	Phe	Arg	Leu	Arg	Ser	
			100					105					110			
AGG	TTT	TAC	CAG	GAA	AAG	GAA	GGA	AGG	GAG	ATA	GAA	GTT	GAG	GGG	GGA	444
Arg	Phe	Tyr	Gln	Glu	Lys	Glu	Gly	Arg	Glu	Ile	Glu	Val	Glu	Gly	Gly	
		115					120					125				
ACG	CTA	AAA	AGC	GTC	AGA	AGA	GTA	GGA	ATG	GAA	GTT	GGG	ACG	GAA	GTT	492
Thr	Leu	Lys	Ser	Val	Arg	Arg	Val	Gly	Met	Glu	Val	Gly	Thr	Glu	Val	
	130					135						140				
GAG	GTT	TAC	GAC	CTC	TTT	TTT	AAC	CTC	CCC	GCA	AGG	AAG	AAA	TTT	TTA	540
Glu	Val	Tyr	Asp	Leu	Phe	Phe	Asn	Leu	Pro	Ala	Arg	Lys	Lys	Phe	Leu	
	145				150					155					160	
AGA	AAG	GAA	GAC	ACC	GAA	AGG	AGA	AAG	ATA	ACG	GAG	CTC	GTA	AAG	GAG	588
Arg	Lys	Glu	Asp	Thr	Glu	Arg	Arg	Lys	Ile	Thr	Glu	Leu	Val	Lys	Glu	
				165					170					175		
TAT	GCC	ATA	ACA	AAC	CCC	CAG	GTT	GAC	TTT	CAC	CTC	TTT	TCC	GAA	GGA	636
Tyr	Ala	Ile	Thr	Asn	Pro	Gln	Val	Asp	Phe	His	Leu	Phe	Ser	Glu	Gly	
			180				185						190			
AAG	GAA	ACC	CTT	AAC	CTG	AAG	AAG	AAG	GAC	CTA	AAA	GGG	AGA	ATT	GAG	684
Lys	Glu	Thr	Leu	Asn	Leu	Lys	Lys	Lys	Asp	Leu	Lys	Gly	Arg	Ile	Glu	
		195					200					205				
GAA	ATC	TTT	GAG	TCA	ATT	TTT	GAA	GAA	GAA	AGC	TCG	GAA	AGG	GAA	GGA	732
Glu	Ile	Phe	Glu	Ser	Ile	Phe	Glu	Glu	Glu	Ser	Ser	Glu	Arg	Glu	Gly	
	210					215					220					
ATA	AAG	GTA	AGA	GCC	TTC	ATA	TCA	AGA	AAC	CAG	AAA	AGG	GGA	AAG	TAT	780
Ile	Lys	Val	Arg	Ala	Phe	Ile	Ser	Arg	Asn	Gln	Lys	Arg	Gly	Lys	Tyr	
	225				230					235					240	
TAC	CTC	TTC	GTA	AAC	TCA	AGA	CCA	GTT	TAC	AAC	AAA	AAC	TTA	AAA	GAA	828
Tyr	Leu	Phe	Val	Asn	Ser	Arg	Pro	Val	Tyr	Asn	Lys	Asn	Leu	Lys	Glu	
				245					250					255		
TAC	CTA	AAG	AAA	ACC	TTC	GGT	TAT	AAA	ACG	ATA	GTC	GTG	CTG	TTC	ATT	876
Tyr	Leu	Lys	Lys	Thr	Phe	Gly	Tyr	Lys	Thr	Ile	Val	Val	Leu	Phe	Ile	

260										265										270											
GAT	ATT	CCC	CCC	TTT	CTC	GTT	GAC	TTT	AAC	GTT	CAC	CCC	AAA	AAG	AAA																924
Asp	Ile	Pro	Pro	Phe	Leu	Val	Asp	Phe	Asn	Val	His	Pro	Lys	Lys	Lys																
		275					280					285																			
GAG	GTA	AAG	TTT	TTA	AAA	GAG	CGA	AAG	ATT	TAC	GAA	CTC	ATA	AGG	GAA																972
Glu	Val	Lys	Phe	Leu	Lys	Glu	Arg	Lys	Ile	Tyr	Glu	Leu	Ile	Arg	Glu																
		290					295					300																			
CTC	TCT	TCC	AGA	AAA	CAC	ACA	ATC	CTT	GAG	ATA	CCT	ACA	CTT	AAT	CAG																1020
Leu	Ser	Ser	Arg	Lys	His	Thr	Ile	Leu	Glu	Ile	Pro	Thr	Leu	Asn	Gln																
					310					315					320																
AAA	ACC	GAA	AGT	TAT	AAA	CCG	ACA	TAC	GAG	GTT	ATA	GGT	CAA	CTA	AAC																1068
Lys	Thr	Glu	Ser	Tyr	Lys	Pro	Thr	Tyr	Glu	Val	Ile	Gly	Gln	Leu	Asn																
				325					330				335																		
GAA	ACC	TTT	ATT	CTC	GTA	AGC	GAC	GGG	AAC	TTT	TTA	TAC	TTC	ATA	GAC																1116
Glu	Thr	Phe	Ile	Leu	Val	Ser	Asp	Gly	Asn	Phe	Leu	Tyr	Phe	Ile	Asp																
			340					345					350																		
CAG	CAC	CTT	CTT	GAT	GAG	AGA	ATA	AAC	TAC	GAG	AAA	AAT	GGA	AAC	GAA																1164
Gln	His	Leu	Leu	Asp	Glu	Arg	Ile	Asn	Tyr	Glu	Lys	Asn	Gly	Asn	Glu																
			355				360					365																			
GAA	CTT	GCC	TGC	AGA	ATT	TCC	GTA	AAA	GCG	GGG	GAA	AAA	TTA	ACA	AAC																1212
Glu	Leu	Ala	Cys	Arg	Ile	Ser	Val	Lys	Ala	Gly	Lys	Lys	Leu	Thr	Asn																
		370					375					380																			
GAA	AAG	ATA	AAA	GAA	CTC	ATA	AAG	GAA	TGG	AAA	AAG	CTT	GAA	AAC	CCC																1260
Glu	Lys	Ile	Lys	Glu	Leu	Ile	Lys	Glu	Trp	Lys	Lys	Leu	Glu	Asn	Pro																
					390				395						400																
CAC	GTA	TGT	CCC	CAC	GGC	AGA	CCT	ATA	TAC	TAC	AAA	CTC	CCC	TTA	AAG																1308
His	Val	Cys	Pro	His	Gly	Arg	Pro	Ile	Tyr	Tyr	Lys	Leu	Pro	Leu	Lys																
				405					410					415																	
GAA	GTA	TAC	GAA	AAG	CTC	GGA	AGG	AGT	TTT	TAAGGTAAAA	TTCTATAGAC																				1358
Glu	Val	Tyr	Glu	Lys	Leu	Gly	Arg	Ser	Phe																						
			420					425																							
CCAATGTTCA GCATTAAGTT CT																														1380	

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 426 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Met Phe Val Lys Ile Leu Pro Pro Glu Val Arg Arg Lys Ile Ala Ala
 1 5 10 15

Gly Glu Val Ile Asp Ala Pro Val Asp Val Val Lys Glu Leu Ile Glu
 20 25 30
 Asn Ser Leu Asp Ala Lys Ala Thr Arg Ile Glu Ile Glu Val Val Lys
 35 40 45
 Gly Gly Lys Arg Leu Ile Arg Val Lys Asp Asn Gly Ile Gly Ile His
 50 55 60
 Pro Glu Asp Ile Glu Lys Val Val Leu Ser Gly Ala Thr Ser Lys Ile
 65 70 75 80
 Glu Lys Glu Thr Asp Leu Leu Asn Val Glu Thr Tyr Gly Phe Arg Gly
 85 90 95
 Glu Ala Leu Tyr Ser Ile Ser Ser Val Ser Lys Phe Arg Leu Arg Ser
 100 105 110
 Arg Phe Tyr Gln Glu Lys Glu Gly Arg Glu Ile Glu Val Glu Gly Gly
 115 120 125
 Thr Leu Lys Ser Val Arg Arg Val Gly Met Glu Val Gly Thr Glu Val
 130 135 140
 Glu Val Tyr Asp Leu Phe Phe Asn Leu Pro Ala Arg Lys Lys Phe Leu
 145 150 155 160
 Arg Lys Glu Asp Thr Glu Arg Arg Lys Ile Thr Glu Leu Val Lys Glu
 165 170 175
 Tyr Ala Ile Thr Asn Pro Gln Val Asp Phe His Leu Phe Ser Glu Gly
 180 185 190
 Lys Glu Thr Leu Asn Leu Lys Lys Lys Asp Leu Lys Gly Arg Ile Glu
 195 200 205
 Glu Ile Phe Glu Ser Ile Phe Glu Glu Glu Ser Ser Glu Arg Glu Gly
 210 215 220
 Ile Lys Val Arg Ala Phe Ile Ser Arg Asn Gln Lys Arg Gly Lys Tyr
 225 230 235 240
 Tyr Leu Phe Val Asn Ser Arg Pro Val Tyr Asn Lys Asn Leu Lys Glu
 245 250 255
 Tyr Leu Lys Lys Thr Phe Gly Tyr Lys Thr Ile Val Val Leu Phe Ile
 260 265 270
 Asp Ile Pro Pro Phe Leu Val Asp Phe Asn Val His Pro Lys Lys Lys
 275 280 285
 Glu Val Lys Phe Leu Lys Glu Arg Lys Ile Tyr Glu Leu Ile Arg Glu
 290 295 300
 Leu Ser Ser Arg Lys His Thr Ile Leu Glu Ile Pro Thr Leu Asn Gln
 305 310 315 320
 Lys Thr Glu Ser Tyr Lys Pro Thr Tyr Glu Val Ile Gly Gln Leu Asn
 325 330 335
 Glu Thr Phe Ile Leu Val Ser Asp Gly Asn Phe Leu Tyr Phe Ile Asp
 340 345 350

Gln	His	Leu	Leu	Asp	Glu	Arg	Ile	Asn	Tyr	Glu	Lys	Asn	Gly	Asn	Glu
		355					360					365			
Glu	Leu	Ala	Cys	Arg	Ile	Ser	Val	Lys	Ala	Gly	Glu	Lys	Leu	Thr	Asn
	370					375					380				
Glu	Lys	Ile	Lys	Glu	Leu	Ile	Lys	Glu	Trp	Lys	Lys	Leu	Glu	Asn	Pro
385					390					395					400
His	Val	Cys	Pro	His	Gly	Arg	Pro	Ile	Tyr	Tyr	Lys	Leu	Pro	Leu	Lys
				405					410					415	
Glu	Val	Tyr	Glu	Lys	Leu	Gly	Arg	Ser	Phe						
			420					425							

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1640 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

```
(ix) FEATURE:
      (A) NAME/KEY: CDS
      (B) LOCATION: 51..1583
```

TTTTTCTGG ATGTTAAAT TTTCAGGGAG ATCGAGTGA GAGGTGTTCT GTT TTG															56	
Val Leu 1																
AGA	ATA	AAA	AGA	CTT	CCC	GAG	AGC	CTC	GTC	AGA	AAA	ATC	GCC	GCG	GGT	104
Arg	Ile	Lys	Arg	Leu	Pro	Glu	Ser	Leu	Val	Arg	Lys	Ile	Ala	Ala	Gly	
		5					10					15				
GAG	GTG	ATT	CAC	AAT	CCA	TCT	TTC	GTT	CTG	AAA	GAG	CTT	GTA	GAA	AAC	152
Glu	Val	Ile	His	Asn	Pro	Ser	Phe	Val	Leu	Lys	Glu	Leu	Val	Glu	Asn	
		20					25					30				
AGT	CTG	GAC	GCG	CAG	GCC	GAC	AGG	ATA	GTT	GTT	GAG	ATA	GAA	AAC	GGT	200
Ser	Leu	Asp	Ala	Gln	Ala	Asp	Arg	Ile	Val	Val	Glu	Ile	Glu	Asn	Gly	
		35			40					45				50		
GGA	AAG	AAC	ATG	GTA	AGA	GTA	TCC	GAC	AAT	GGA	ATC	GGG	ATG	ACC	AGA	248
Gly	Lys	Asn	Met	Val	Arg	Val	Ser	Asp	Asn	Gly	Ile	Gly	Met	Thr	Arg	
				55					60					65		
GAA	GAG	GCA	CTT	CTG	GCA	ATA	GAA	CCT	TAC	ACG	ACG	AGC	AAG	ATA	GAG	296
Glu	Glu	Ala	Leu	Leu	Ala	Ile	Glu	Pro	Tyr	Thr	Thr	Ser	Lys	Ile	Glu	
				70					75					80		
AGC	GAG	GAA	GAT	CTG	CAC	AGG	ATC	AGA	ACT	TAC	GGT	TTC	AGA	GGT	GAA	344
Ser	Glu	Glu	Asp	Leu	His	Arg	Ile	Arg	Thr	Tyr	Gly	Phe	Arg	Gly	Glu	

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85					90					95						
GCG	CTT	GCT	TCG	ATT	GTG	CAG	GTC	AGC	AGA	GCC	AAG	ATC	GTG	ACA	AAA	392
Ala	Leu	Ala	Ser	Ile	Val	Gln	Val	Ser	Arg	Ala	Lys	Ile	Val	Thr	Lys	
100						105				110						
ACG	GAA	AAA	GAC	GCA	CTC	GCA	ACA	CAG	TTG	ATG	ATT	GCT	GGG	GGG	AAA	440
Thr	Glu	Lys	Asp	Ala	Leu	Ala	Thr	Gln	Leu	Met	Ile	Ala	Gly	Gly	Lys	
115					120					125					130	
GTG	GAA	GAA	ATC	TCG	GAA	ACC	CAC	AGG	GAT	ACC	GGC	ACC	ACC	GTT	GAG	488
Val	Glu	Glu	Ile	Ser	Glu	Thr	His	Arg	Asp	Thr	Gly	Thr	Thr	Val	Glu	
				135					140					145		
GTG	AGA	GAT	CTC	TTC	TTC	AAC	CTA	CCC	GTC	CGG	AGA	AAA	TCT	CTG	AAG	536
Val	Arg	Asp	Leu	Phe	Phe	Asn	Leu	Pro	Val	Arg	Arg	Lys	Ser	Leu	Lys	
			150					155					160			
TCC	TCT	GCC	ATC	GAG	TTG	AGA	ATG	TGT	CGT	GAG	ATG	TTT	GAA	AGA	TTC	584
Ser	Ser	Ala	Ile	Glu	Leu	Arg	Met	Cys	Arg	Glu	Met	Phe	Glu	Arg	Phe	
		165					170					175				
GTC	CTT	GTA	CGA	AAC	GAC	GTT	GAT	TTT	GTA	TTC	ACC	TCA	GAT	GGA	AAG	632
Val	Leu	Val	Arg	Asn	Asp	Val	Asp	Phe	Val	Phe	Thr	Ser	Asp	Gly	Lys	
	180					185					190					
ATA	GTC	CAT	TCC	TTT	CCA	AGA	ACA	CAG	AAC	ATC	TTT	GAA	AGA	GCT	CTC	680
Ile	Val	His	Ser	Phe	Pro	Arg	Thr	Gln	Asn	Ile	Phe	Glu	Arg	Ala	Leu	
195					200					205					210	
CTG	ATC	CTT	GAA	GAT	CTG	AGA	AAA	GGT	TAC	ATC	ACG	TTC	GAA	GAG	GAA	728
Leu	Ile	Leu	Glu	Asp	Leu	Arg	Lys	Gly	Tyr	Ile	Thr	Phe	Glu	Glu	Glu	
				215				220						225		
TTA	TCC	GGC	CTG	AGG	ATA	AAG	GGA	ATA	GTT	TCA	TCC	CGC	GAG	GTG	ACA	776
Leu	Ser	Gly	Leu	Arg	Ile	Lys	Gly	Ile	Val	Ser	Ser	Arg	Glu	Val	Thr	
			230					235					240			
AGA	TCC	AGC	AGA	ACG	GGA	GAG	TAT	TTC	TAC	GTG	AAC	GGT	CGT	TTT	GTG	824
Arg	Ser	Ser	Arg	Thr	Gly	Glu	Tyr	Phe	Tyr	Val	Asn	Gly	Arg	Phe	Val	
		245					250					255				
GTT	TCC	GAA	GAA	CTC	CAC	GAA	GTA	CTC	ATG	AAA	GTT	TAC	GAT	CTT	CCA	872
Val	Ser	Glu	Glu	Leu	His	Glu	Val	Leu	Met	Lys	Val	Tyr	Asp	Leu	Pro	
		260				265					270					
AAG	AGA	AGC	TAT	CCC	GTC	GCG	GTT	CTT	TTC	ATA	GAG	GTA	AAT	CCG	GAA	920
Lys	Arg	Ser	Tyr	Pro	Val	Ala	Val	Leu	Phe	Ile	Glu	Val	Asn	Pro	Glu	
275					280					285					290	
GAA	CTC	GAC	GTG	AAC	ATA	CAC	CCT	TCG	AAA	ATC	GTG	GTG	AAA	TTT	CTC	968
Glu	Leu	Asp	Val	Asn	Ile	His	Pro	Ser	Lys	Ile	Val	Val	Lys	Phe	Leu	
				295					300					305		
AAC	GAA	GAA	AAG	GTG	AAA	AAG	AGT	TTG	GAA	GAA	ACC	CTC	AAA	AGA	AAT	1016
Asn	Glu	Glu	Lys	Val	Lys	Lys	Ser	Leu	Glu	Glu	Thr	Leu	Lys	Arg	Asn	
			310					315					320			
CTG	GCA	CGG	AAA	TGG	TAC	AGG	TCG	GTT	GCG	TAC	GAA	GAA	ATA	TCC	TCC	1064
Leu	Ala	Arg	Lys	Trp	Tyr	Arg	Ser	Val	Ala	Tyr	Glu	Glu	Ile	Ser	Ser	
		325					330						335			

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CGT	GCG	CTG	AGC	GTG	GCA	GAA	GCA	CCA	TCC	CAC	AGA	TGG	TTT	TTG	GTC	1112
Arg	Ala	Leu	Ser	Val	Ala	Glu	Ala	Pro	Ser	His	Arg	Trp	Phe	Leu	Val	
340						345					350					
AAG	GGT	AAG	TAC	GCT	GTC	GTT	GAA	GTG	GAA	GAT	GGT	TTG	CTC	TTT	GTG	1160
Lys	Gly	Lys	Tyr	Ala	Val	Val	Glu	Val	Glu	Asp	Gly	Leu	Leu	Phe	Val	
355					360					365					370	
GAT	CTT	CAT	GCT	CTC	CAC	GAA	CGA	ACG	ATT	TAC	GAA	GAA	ATC	CTT	TCG	1208
Asp	Leu	His	Ala	Leu	His	Glu	Arg	Thr	Ile	Tyr	Glu	Glu	Ile	Leu	Ser	
				375					380					385		
AAA	AAA	AGC	TGG	GGG	AAA	AGA	CGG	GTG	AAA	AGG	AAC	ATA	ACA	GTT	GTG	1256
Lys	Lys	Ser	Trp	Gly	Lys	Arg	Arg	Val	Lys	Arg	Asn	Ile	Thr	Val	Val	
			390					395					400			
CTA	TCA	AGG	GAA	GAA	AAA	CAA	AAA	CTG	GAA	GAA	TAC	GGA	TTC	TCC	TTT	1304
Leu	Ser	Arg	Glu	Glu	Lys	Gln	Lys	Leu	Glu	Glu	Tyr	Gly	Phe	Ser	Phe	
		405					410					415				
CAA	GGA	GAA	GAA	GGA	GCT	TTG	AAA	GTC	ATT	GAA	ATC	CCT	GAG	TTC	CTC	1352
Gln	Gly	Glu	Glu	Gly	Ala	Leu	Lys	Val	Ile	Glu	Ile	Pro	Glu	Phe	Leu	
	420					425					430					
ACC	GAA	GAC	GTT	GTG	GAG	GAA	TTT	TTC	AGG	GAC	TTC	CCA	GTT	GAT	GAA	1400
Thr	Glu	Asp	Val	Val	Glu	Glu	Phe	Phe	Arg	Asp	Phe	Pro	Val	Asp	Glu	
435					440					445					450	
AAA	CTG	AAG	GAA	AGA	ATA	GCC	CTT	GCC	GCT	TGT	AAA	CTT	GCC	ACT	AAA	1448
Lys	Leu	Lys	Glu	Arg	Ile	Ala	Leu	Ala	Ala	Cys	Lys	Leu	Ala	Thr	Lys	
				455					460					465		
TCC	GGA	GAA	TTC	GAC	GAA	GAG	ATC	GCA	TCG	AAA	CTG	CTG	GAT	GTC	TTT	1496
Ser	Gly	Glu	Phe	Asp	Glu	Glu	Ile	Ala	Ser	Lys	Leu	Leu	Asp	Val	Phe	
			470					475					480			
TTC	AAG	AAG	CGG	TTT	GAA	AGA	TGT	CCT	CAC	GGA	AGG	CCG	ATT	TCT	TTC	1544
Phe	Lys	Lys	Arg	Phe	Glu	Arg	Cys	Pro	His	Gly	Arg	Pro	Ile	Ser	Phe	
		485					490					495				
AAG	ATC	AGC	TAT	GAG	GAC	ATG	GAC	CGA	TTT	TTC	GAG	CGT	TAACCCATTT			1593
Lys	Ile	Ser	Tyr	Glu	Asp	Met	Asp	Arg	Phe	Phe	Glu	Arg				
	500					505					510					
TCACCACGTT	GACGTCAGCG	GTGAAAACCA	GGCCATCGAA	GTCTATG												1640

(2) INFORMATION FOR SEQ ID NO:42:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 511 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Val Leu Arg Ile Lys Arg Leu Pro Glu Ser Leu Val Arg Lys Ile Ala

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1	5	10	15
Ala Gly Glu Val	Ile His Asn Pro Ser Phe Val Leu Lys Glu Leu Val		
20		25	30
Glu Asn Ser Leu Asp Ala Gln Ala Asp Arg Ile Val Val Glu Ile Glu			
35		40	45
Asn Gly Gly Lys Asn Met Val Arg Val Ser Asp Asn Gly Ile Gly Met			
50		55	60
Thr Arg Glu Glu Ala Leu Leu Ala Ile Glu Pro Tyr Thr Thr Ser Lys			
65	70	75	80
Ile Glu Ser Glu Glu Asp Leu His Arg Ile Arg Thr Tyr Gly Phe Arg			
	85	90	95
Gly Glu Ala Leu Ala Ser Ile Val Gln Val Ser Arg Ala Lys Ile Val			
100		105	110
Thr Lys Thr Glu Lys Asp Ala Leu Ala Thr Gln Leu Met Ile Ala Gly			
115		120	125
Gly Lys Val Glu Glu Ile Ser Glu Thr His Arg Asp Thr Gly Thr Thr			
130		135	140
Val Glu Val Arg Asp Leu Phe Phe Asn Leu Pro Val Arg Arg Lys Ser			
145		150	155
Leu Lys Ser Ser Ala Ile Glu Leu Arg Met Cys Arg Glu Met Phe Glu			
	165	170	175
Arg Phe Val Leu Val Arg Asn Asp Val Asp Phe Val Phe Thr Ser Asp			
	180	185	190
Gly Lys Ile Val His Ser Phe Pro Arg Thr Gln Asn Ile Phe Glu Arg			
195		200	205
Ala Leu Leu Ile Leu Glu Asp Leu Arg Lys Gly Tyr Ile Thr Phe Glu			
210		215	220
Glu Glu Leu Ser Gly Leu Arg Ile Lys Gly Ile Val Ser Ser Arg Glu			
225		230	235
Val Thr Arg Ser Ser Arg Thr Gly Glu Tyr Phe Tyr Val Asn Gly Arg			
	245	250	255
Phe Val Val Ser Glu Glu Leu His Glu Val Leu Met Lys Val Tyr Asp			
	260	265	270
Leu Pro Lys Arg Ser Tyr Pro Val Ala Val Leu Phe Ile Glu Val Asn			
275		280	285
Pro Glu Glu Leu Asp Val Asn Ile His Pro Ser Lys Ile Val Val Lys			
290		295	300
Phe Leu Asn Glu Glu Lys Val Lys Lys Ser Leu Glu Glu Thr Leu Lys			
305		310	315
Arg Asn Leu Ala Arg Lys Trp Tyr Arg Ser Val Ala Tyr Glu Glu Ile			
	325	330	335

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Ser Ser Arg Ala Leu Ser Val Ala Glu Ala Pro Ser His Arg Trp Phe
 340 345 350
 Leu Val Lys Gly Lys Tyr Ala Val Val Glu Val Glu Asp Gly Leu Leu
 355 360 365
 Phe Val Asp Leu His Ala Leu His Glu Arg Thr Ile Tyr Glu Glu Ile
 370 375 380
 Leu Ser Lys Lys Ser Trp Gly Lys Arg Arg Val Lys Arg Asn Ile Thr
 385 390 395 400
 Val Val Leu Ser Arg Glu Glu Lys Gln Lys Leu Glu Glu Tyr Gly Phe
 405 410 415
 Ser Phe Gln Gly Glu Glu Gly Ala Leu Lys Val Ile Glu Ile Pro Glu
 420 425 430
 Phe Leu Thr Glu Asp Val Val Glu Phe Phe Arg Asp Phe Pro Val
 435 440 445
 Asp Glu Lys Leu Lys Glu Arg Ile Ala Leu Ala Ala Cys Lys Leu Ala
 450 455 460
 Thr Lys Ser Gly Glu Phe Asp Glu Glu Ile Ala Ser Lys Leu Leu Asp
 465 470 475 480
 Val Phe Phe Lys Lys Arg Phe Glu Arg Cys Pro His Gly Arg Pro Ile
 485 490 495
 Ser Phe Lys Ile Ser Tyr Glu Asp Met Asp Arg Phe Phe Glu Arg
 500 505 510

(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 649 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Met Ser His Ile Ile Glu Leu Pro Glu Met Leu Ala Asn Gln Ile Ala
 1 5 10 15
 Ala Gly Glu Val Ile Glu Arg Pro Ala Ser Val Cys Lys Glu Leu Val
 20 25 30
 Glu Asn Ala Ile Asp Ala Gly Ser Ser Gln Ile Ile Ile Glu Ile Glu
 35 40 45
 Glu Ala Gly Leu Lys Lys Val Gln Ile Thr Asp Asn Gly His Gly Ile
 50 55 60
 Ala His Asp Glu Val Glu Leu Ala Leu Arg Arg His Ala Thr Ser Lys

65	70	75	80
Ile Lys Asn Gln Ala Asp Leu Phe Arg	Ile Arg Thr Leu Gly Phe Arg		
85	90		95
Gly Glu Ala Leu Pro Ser Ile Ala Ser Val Ser Val Leu Thr Leu Leu			
100	105		110
Thr Ala Val Asp Gly Ala Ser His Gly Thr Lys Leu Val Ala Arg Gly			
115	120		125
Gly Glu Val Glu Glu Val Ile Pro Ala Thr Ser Pro Val Gly Thr Lys			
130	135	140	
Val Cys Val Glu Asp Leu Phe Phe Asn Thr Pro Ala Arg Leu Lys Tyr			
145	150	155	160
Met Lys Ser Gln Gln Ala Glu Leu Ser His Ile Ile Asp Ile Val Asn			
165	170		175
Arg Leu Gly Leu Ala His Pro Glu Ile Ser Phe Ser Leu Ile Ser Asp			
180	185		190
Gly Lys Glu Met Thr Arg Thr Ala Gly Thr Gly Gln Leu Arg Gln Ala			
195	200		205
Ile Ala Gly Ile Tyr Gly Leu Val Ser Ala Lys Lys Met Ile Glu Ile			
210	215	220	
Glu Asn Ser Asp Leu Asp Phe Glu Ile Ser Gly Phe Val Ser Leu Pro			
225	230	235	240
Glu Leu Thr Arg Ala Asn Arg Asn Tyr Ile Ser Leu Phe Ile Asn Gly			
245	250		255
Arg Tyr Ile Lys Asn Phe Leu Leu Asn Arg Ala Ile Leu Asp Gly Phe			
260	265		270
Gly Ser Lys Leu Met Val Gly Arg Phe Pro Leu Ala Val Ile His Ile			
275	280		285
His Ile Asp Pro Tyr Leu Ala Asp Val Asn Val His Pro Thr Lys Gln			
290	295	300	
Glu Val Arg Ile Ser Lys Glu Lys Glu Leu Met Thr Leu Val Ser Glu			
305	310	315	320
Ala Ile Ala Asn Ser Leu Lys Glu Gln Thr Leu Ile Pro Asp Ala Leu			
325	330		335
Glu Asn Leu Ala Lys Ser Thr Val Arg Asn Arg Glu Lys Val Glu Gln			
340	345		350
Thr Ile Leu Pro Leu Lys Glu Asn Thr Leu Tyr Tyr Glu Lys Thr Glu			
355	360		365
Pro Ser Arg Pro Ser Gln Thr Glu Val Ala Asp Tyr Gln Val Glu Leu			
370	375	380	
Thr Asp Glu Gly Gln Asp Leu Thr Leu Phe Ala Lys Glu Thr Leu Asp			
385	390	395	400

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Arg Leu Thr Lys Pro Ala Lys Leu His Phe Ala Glu Arg Lys Pro Ala
      405                               410                      415
Asn Tyr Asp Gln Leu Asp His Pro Glu Leu Asp Leu Ala Ser Ile Asp
      420                               425                      430
Lys Ala Tyr Asp Lys Leu Glu Arg Glu Glu Ala Ser Ser Phe Pro Glu
      435                               440                      445
Leu Glu Phe Phe Gly Gln Met His Gly Thr Tyr Leu Phe Ala Gln Gly
      450                               455                      460
Arg Asp Gly Leu Tyr Ile Ile Asp Gln His Ala Ala Gln Glu Arg Val
      465                               470                      475                      480
Lys Tyr Glu Glu Tyr Arg Glu Ser Ile Gly Asn Val Asp Gln Ser Gln
      485                               490                      495
Gln Gln Leu Leu Val Pro Tyr Ile Phe Glu Phe Pro Ala Asp Asp Ala
      500                               505                      510
Leu Arg Leu Lys Glu Arg Met Pro Leu Leu Glu Glu Val Gly Val Phe
      515                               520                      525
Leu Ala Glu Tyr Gly Glu Asn Gln Phe Ile Leu Arg Glu His Pro Ile
      530                               535                      540
Trp Met Ala Glu Glu Glu Ile Glu Ser Gly Ile Tyr Glu Met Cys Asp
      545                               550                      555                      560
Met Leu Leu Leu Thr Lys Glu Val Ser Ile Lys Lys Tyr Arg Ala Glu
      565                               570                      575
Leu Ala Ile Met Met Ser Cys Lys Arg Ser Ile Lys Ala Asn His Arg
      580                               585                      590
Ile Asp Asp His Ser Ala Arg Gln Leu Leu Tyr Gln Leu Ser Gln Cys
      595                               600                      605
Asp Asn Pro Tyr Asn Cys Pro His Gly Arg Pro Val Leu Val His Phe
      610                               615                      620
Thr Lys Ser Asp Met Glu Lys Met Phe Arg Arg Ile Gln Glu Asn His
      625                               630                      635                      640
Thr Ser Leu Arg Glu Leu Gly Lys Tyr
      645

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(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 615 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Met	Pro	Ile	Gln	Val	Leu	Pro	Pro	Gln	Leu	Ala	Asn	Gln	Ile	Ala	Ala	1	5	10	15
Gly	Glu	Val	Val	Glu	Arg	Pro	Ala	Ser	Val	Val	Lys	Glu	Leu	Val	Glu	20	25	30	
Asn	Ser	Leu	Asp	Ala	Gly	Ala	Thr	Arg	Ile	Asp	Ile	Asp	Ile	Glu	Arg	35	40	45	
Gly	Gly	Ala	Lys	Leu	Ile	Arg	Ile	Arg	Asp	Asn	Gly	Cys	Gly	Ile	Lys	50	55	60	
Lys	Asp	Glu	Leu	Ala	Leu	Ala	Leu	Ala	Arg	His	Ala	Thr	Ser	Lys	Ile	65	70	75	80
Ala	Ser	Leu	Asp	Asp	Leu	Glu	Ala	Ile	Ile	Ser	Leu	Gly	Phe	Arg	Gly	85	90	95	
Glu	Ala	Leu	Ala	Ser	Ile	Ser	Ser	Val	Ser	Arg	Leu	Thr	Leu	Thr	Ser	100	105	110	
Arg	Thr	Ala	Glu	Gln	Gln	Glu	Ala	Trp	Gln	Ala	Tyr	Ala	Glu	Gly	Arg	115	120	125	
Asp	Met	Asn	Val	Thr	Val	Lys	Pro	Ala	Ala	His	Pro	Val	Gly	Thr	Thr	130	135	140	
Leu	Glu	Val	Leu	Asp	Leu	Phe	Tyr	Asn	Thr	Pro	Ala	Arg	Arg	Lys	Phe	145	150	155	160
Leu	Arg	Thr	Glu	Lys	Thr	Glu	Phe	Asn	His	Ile	Asp	Glu	Ile	Ile	Arg	165	170	175	
Arg	Ile	Ala	Leu	Ala	Arg	Phe	Asp	Val	Thr	Ile	Asn	Leu	Ser	His	Asn	180	185	190	
Gly	Lys	Ile	Val	Arg	Gln	Tyr	Arg	Ala	Val	Pro	Glu	Gly	Gly	Gln	Lys	195	200	205	
Glu	Arg	Arg	Leu	Gly	Ala	Ile	Cys	Gly	Thr	Ala	Phe	Leu	Glu	Gln	Ala	210	215	220	
Leu	Ala	Ile	Glu	Trp	Gln	His	Gly	Asp	Leu	Thr	Leu	Arg	Gly	Trp	Val	225	230	235	240
Ala	Asp	Pro	Asn	His	Thr	Thr	Pro	Ala	Leu	Ala	Glu	Ile	Gln	Tyr	Cys	245	250	255	
Tyr	Val	Asn	Gly	Arg	Met	Met	Arg	Asp	Arg	Leu	Ile	Asn	His	Ala	Ile	260	265	270	
Arg	Gln	Ala	Cys	Glu	Asp	Lys	Leu	Gly	Ala	Asp	Gln	Gln	Pro	Ala	Phe	275	280	285	
Val	Leu	Tyr	Leu	Glu	Ile	Asp	Pro	His	Gln	Val	Asp	Val	Asn	Val	His	290	295	300	
Pro	Ala	Lys	His	Glu	Val	Arg	Phe	His	Gln	Ser	Arg	Leu	Val	His	Asp	305	310	315	320

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Phe Ile Tyr Gln Gly Val Leu Ser Val Leu Gln Gln Gln Leu Glu Thr
 325 330 335
 Pro Leu Pro Leu Asp Asp Glu Pro Gln Pro Ala Pro Arg Ser Ile Pro
 340 345 350
 Glu Asn Arg Val Ala Ala Gly Arg Asn His Phe Ala Glu Pro Ala Ala
 355 360 365
 Arg Glu Pro Val Ala Pro Arg Tyr Thr Pro Ala Pro Ala Ser Gly Ser
 370 375 380
 Arg Pro Ala Ala Pro Trp Pro Asn Ala Gln Pro Gly Tyr Gln Lys Gln
 385 390 395 400
 Gln Gly Glu Val Tyr Arg Gln Leu Leu Gln Thr Pro Ala Pro Met Gln
 405 410 415
 Lys Leu Lys Ala Pro Glu Pro Gln Glu Pro Ala Leu Ala Ala Asn Ser
 420 425 430
 Gln Ser Phe Gly Arg Val Leu Thr Ile Val His Ser Asp Cys Ala Leu
 435 440 445
 Leu Glu Arg Asp Gly Asn Ile Ser Leu Leu Ser Leu Pro Val Ala Glu
 450 455 460
 Arg Trp Leu Arg Gln Ala Gln Leu Thr Pro Gly Glu Ala Pro Val Cys
 465 470 475 480
 Ala Gln Pro Leu Leu Ile Pro Leu Arg Leu Lys Val Ser Ala Glu Glu
 485 490 495
 Lys Ser Ala Leu Glu Lys Ala Gln Ser Ala Leu Ala Glu Leu Gly Ile
 500 505 510
 Asp Phe Gln Ser Asp Ala Gln His Val Thr Ile Arg Ala Val Pro Leu
 515 520 525
 Pro Leu Arg Gln Gln Asn Leu Gln Ile Leu Ile Pro Glu Leu Ile Gly
 530 535 540
 Tyr Leu Ala Lys Gln Ser Val Phe Glu Pro Gly Asn Ile Ala Gln Trp
 545 550 555 560
 Ile Ala Arg Asn Leu Met Ser Glu His Ala Gln Trp Ser Met Ala Gln
 565 570 575
 Ala Ile Thr Leu Leu Ala Asp Val Glu Arg Leu Cys Pro Gln Leu Val
 580 585 590
 Lys Thr Pro Pro Gly Gly Leu Leu Gln Ser Val Asp Leu His Pro Ala
 595 600 605
 Ile Lys Ala Leu Lys Asp Glu
 610 615

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 396 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GAATTCGATC ACCTGCAAGA AGTCATCAAG CGCCTGGCCC TGGCCCGTTT CGACGTGGCC	60
TTTCACCTGC GCCACAATGG CAAGACCATC CTCAGCCTGC ACGAAGCCAA CGACGACGCC	120
GCCCGTGCTC GGCGGGTGGC GGCGGTGTGT GGCAGCGGGT TCCTGGAGCA GGCCTGCGCC	180
ATTGAGATCG AGCGCAATGG CTTGAGGTTG TGGGGCTGGG TCGGGTTGCC GACGTTCTCC	240
CGCAGCCAGG CCGATTTGCA GTATTTCTTT GTGAACGGCC GGGCGGTCCG CGACAAACTG	300
GTGGCCCATG CCGTGCGCCA GGCTTATCGC GATGTGCTGT TCAACGGGCG ACACCCGACT	360
TTTGTGCTGT TCTTTGAGGT TGACCCCTTCG GTGGTC	396

CLAIMS

What is claimed is:

1. An isolated protein which enhances specific binding of
a thermostable mismatch binding protein to bulge loops
5 in a heteroduplex nucleic acid.
2. A protein of claim 1 which is obtainable (e.g.
isolated) from the group consisting of:
hyperthermophilic bacteria and thermophilic bacteria.
3. A protein of claim 1 or claim 2 which is MutL.
- 10 4. A protein of any one of the preceding claims having
the amino acid sequence SEQ ID NO: 40 or SEQ ID
NO: 42.
5. A protein of any one of the preceding claims which is
15 encoded by a nucleic acid characterised by the ability
to hybridise to nucleic acid having a sequence
selected from the group consisting of: SEQ ID
NOs: 39, 41 and 45.
6. An isolated nucleic acid which encodes a protein as
defined in any one of the preceding claims.
- 20 7. A nucleic acid of claim 6 which hybridises to nucleic
acid having a sequence selected from the group
consisting of: SEQ ID NOs: 39, 41 and 45.
8. A recombinant vector comprising the nucleic acid of
claim 6 or claim 7.

9. A host cell which: (a) comprises the nucleic acid of claim 6 or claim 7; or (b) comprises the vector of claim 8; or (c) comprises a recombinant gene which can express a protein as defined in any one of claims 1-5; or (d) expresses a protein as defined in any one of claims 1-5 which is heterologous to the host.
10. Use of the protein as defined in any one of claims 1-5 in:
- (a) a method of reducing DNA misincorporation in an amplification reaction (e.g. a ligase or polymerase chain reaction); or
 - (b) a method for detecting a nucleic acid which includes a specific sequence (e.g. a mutation); or
 - (c) an amplification method, e.g. a method for amplifying a nucleic acid comprising a specific sequence; or
 - (d) selecting against a nucleic acid comprising a specific sequence.
11. A method of reducing DNA misincorporation in an amplification reaction (e.g. a ligase or polymerase chain reaction) comprising the step of including a thermostable mismatch binding protein and a protein as defined in any one of claims 1-5 in the reaction.
12. A method for detecting a nucleic acid which includes a specific sequence (e.g. a mutation) comprising the steps of:
- (a) combining a thermostable mismatch binding protein, a thermostable protein that enhances specific binding of the thermostable mismatch binding protein to bulge loops in a heteroduplex

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- nucleic acid, and an amplification reaction mixture, thereby producing a test combination;
- (b) maintaining the test combination of step (a) under conditions appropriate for amplification of nucleic acids to occur, thereby promoting synthesis of extension products;
- (c) determining the amount of product synthesised in step (b); and
- (d) comparing the amount of product determined in step (c) with the amount of product synthesised in a corresponding negative control to determine if the specific sequence suspected of being present in the nucleic acid is present.
13. A method of claim 12 wherein the amplification reaction mixture comprises nucleic acids to be assessed for a specific sequence of interest, four different nucleoside triphosphates, two oligonucleotide primers wherein each primer is selected to be complementary to different strands of the nucleic acid which includes the specific sequence of interest, blocking oligonucleotides which are completely complementary to the specific sequence of interest, a thermostable enzyme which catalyses combination of the nucleoside triphosphates to form primer extension products complementary to each strand of the nucleic acid which includes the specific sequence of interest, and an amplification buffer suitable for the activity of the enzyme.
14. A method for amplifying a nucleic acid comprising a specific sequence comprising the steps of:
- (a) combining a thermostable mismatch binding protein, a thermostable protein that enhances specific binding of the thermostable mismatch

- nucleic acid protein to bulge loops in a heteroduplex nucleic acid, and an amplification reaction mixture, thereby producing a test combination; and
- 5 (b) maintaining the test combination of step (a) under conditions appropriate for amplification of nucleic acids to occur, resulting in synthesis of the nucleic acid comprising the sequence of interest.
- 10 15. A method of claim 14 wherein the amplification reaction mixture comprises a nucleic acid comprising a specific sequence to be amplified, four different nucleoside triphosphates, two oligonucleotide primers wherein each primer is selected to be complementary to
- 15 different strands of the nucleic acid comprising the specific sequence to be amplified, blocking oligonucleotides which form heteroduplexes with a strand of nucleic acids being selected against, a thermostable enzyme which catalyses combination of the
- 20 nucleoside triphosphates to form primer extension products complementary to each strand of the nucleic acid comprising the specific sequence to be amplified, and an amplification buffer suitable for the activity of the enzyme.
- 25 16. A method of selecting against a nucleic acid comprising a specific sequence comprising the steps of:
- 30 (a) combining a thermostable mismatch binding protein, a thermostable protein that enhances specific binding of the thermostable mismatch binding protein to bulge loops in a heteroduplex nucleic acid, and an amplification reaction

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- mixture, thereby producing a test combination;
and
- (b) maintaining the test combination of step (a)
under conditions appropriate for amplification of
5 nucleic acids to occur, thereby selecting against
a nucleic acid comprising the specific sequence.
17. A method of claim 16 wherein the amplification
reaction mixture comprises nucleic acids comprising a
specific sequence to be amplified or detected and
10 nucleic acids whose synthesis is to be prevented or
reduced, four different nucleoside triphosphates, two
oligonucleotide primers wherein each primer is
selected to be complementary to different strands of
the nucleic acids comprising a specific sequence to be
15 amplified or detected, blocking oligonucleotides which
form heteroduplexes with a strand of the nucleic acids
whose synthesis is to be prevented or reduced, a
thermostable enzyme which catalyses combination of the
nucleoside triphosphates to form primer extension
20 products complementary to each strand of the nucleic
acids comprising the specific sequence to be amplified
or detected, and an amplification buffer suitable for
the activity of the enzyme.
18. A method of any one of claims 11-17 further comprising
25 including a stabilizer (e.g. in step (a)).
19. A method of amplification characterised in that a
protein as defined in any one of claims 1-5 is added
to a solution comprising an amplification reaction
mixture and the protein.

20. The method of claim 19 wherein the protein is a MutL protein and the thermostable mismatch binding protein is a thermostable MutS protein.

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Apy MutS CODING SEQUENCE

```

1  ATGGGAAAAG AGGAGAAAAGA GCTCACCCCC ATGCTCGCCC AGTATCACCA
51  GTTCAAGAGC ATGTATCCCG ACTGCCTTCT TTTATTCAGG CTCGGGGGACT
101 TTTACGAGCT CTTTTACGAG GACGCGGTCT TCGGTTCTAA AGAGCTCGGT
151 CTAGTTCTAA CTTCAAGACC CGCGGGAAAG GGAAGGGAAA GGATTCCCAT
201 GTGCGGTGTT CCCTACCATT CTGCAAACAA CTATATAGCA AAGCTCGTTA
251 ATAAGGGATA CAAGGTAGCA ATATGCGAGC AGGTTGAGGA CCCCTCAAAG
301 GCAAAGGGAA TAGTAAAGAG GGACGTAATA AGAGTTATAA CACCTGGGAC
351 CTTTTTTGAG AGGGAAACGG GAGGGCTTTG CTCCCTTTAC AGGAAGGGAA
401 AGAGCTATCT CGTTTCTTAT CTTAACCTCT CGGTAGGTGA GTTCATAGGT
451 GCAAAGGTAA AGGAGGAAGA GCTCATAGAC TTCCTCTCAA AGTTCAACAT
501 AAGGGAGGTT CTTGTAAAGA AGGGAGAAAA GCTCCCCGAA AAGAGGAGTT
551 AGGTTCTAAA GCTCCACATA ACGGAGCTTG AAGAGGAGTT CTTTGAGGAG
601 GGAAGGAGG AGCTTCTTAA GGATTACGGA GTTCCGTCGA TAAAAGCCTT
651 CGGCTTTTCA GATGAGGATT TATCCCTTTC CCTCGGGGCT GTTTACAGGT
701 ATGCAAAGGC GACACAGAAA TCTTTTACCC CTCTCATTCC AAAGCCCCAA
751 CCTTACGTTG ACGAGGGGATA CGTAAAGCTT GACCTCAAGG CAGTCAAAGG
801 TCTTGAGATT ACCGAAAGCA TAGAAGGAAG AAAGGATTTA TCCCTGTTTA
851 AGGTCGTTGA CAGAACCCTC ACGGGTATGG GGAGAAGGAG GCTGAGGTTT
901 AGGCTTCTAA ACCCCTTCAG GAGCATAGAG AGAATAAGGA AGGTTCAGGA
951 AGCAGTTGAG GAGCTAATAA ACAAGAGGGA GGTCTGAAC GAGATAAGGA
1001 AAACCCTTGA GGGTATGTCC GACCTTGAGA GACTCGTATC CAGGATAAGC
1051 TCAAACATGG CAAGCCCAAG AGAACTTATA CACCTCAAAA ACTCCCTAAG
1101 GAAGGCGGAG GAGCTAAGGA AAATTTTATC TTTGCTTGAT TCCGAAATAT
1151 TTAAAGAGAT AGAAGGTTCT CTCCTTAACC TGAATAAAGT TCGGAGCCTC
1201 ATTGATAAAA CGCTTGTTGA CGACCCTCCC CTGCACGTAA AAGAAGGGGG
1251 GCTTATAAAA CCGGGTGTTA ACGCATACCT TGATGAGCTT CGCTTCATAA
1301 GGGAGAAATG GGAAGGCTC CTGAAGGAGT ATGAAAAGAA GCTGAAAAAA
1351 GAAACGGGAA TTCAGAGCTT AAAGATTGGA TACAACAAGG TTATGGGATA
1401 CTACATAGAG GTAACGAAGG CTAACGTAAA ATACGTTCCC GAACACTTCA
1451 GAAGAAGACA GACCCTTTCA AACGCGGAGA GATACACAAC CGAGGAGCTC
1501 CAGAGACTTG AGGAAAAGAT ACTTCCGCC CAGACCCGCA TAAACGAGCT
1551 TGAGTATGAG CTTTACAGGG AGCTCAGGGA AGAGGTTGTT AAGGAGCTTG
1601 ATAAGGTAGG GAATAACGCA ACCCTCATAG GGGAGGTGGA CTACATCCAG
1651 TCCCTCGCCT GGCTTGCCCT TGAGAAGGGA TGGGTAAAGC CGGAAGTTCA
1701 CGAGGGATAT GAGCTGATAA TAGAGGAGGG AAAGCATCCC GTAATAGAGG
1751 AGTTCACGAA AAACACGTC CCAAACGATA CGAAGCTAAC GGAAGAGGAG
1801 TTCATACACG TAATCACGGG CCCTAACATG GCGGGAAAGT CGAGCTACAT
1851 AAGACAGGTG GGCCTCCTCA CGCTCCTTGC TCATACAGGT AGCTTCCTTC
1901 CCGTAAAGAG TGCAAGGATA CCGCTGGTTG ATGCGATATT CACGAGAATA
1951 GGCTCGGGGG ACGTCTGGC TCTGGGTGTT TCAACCTTCA TGAACGAGAT
2001 GCTTGACGTG TCAAACATAC TCAACAACGC AACGAAGAGG AGCTTAATAA
2051 TACTCGACGA GGTGGGAAGG GGAACCTCAA CCTACGACGG GATAGCGATA
2101 AGCAAGGCGA TAGTGAAATA CATAAGCGAG AAGATAGGGG CGAAAACGCT
2151 ACTCGCAACC CACTACCTTG AGCTAACCGA GCTTGAGAGA AAGGTAAAGG
2201 GAGTAAAGAA CTACCACATG GAGGTTGAGG AAACGGATGA GGAATAAGG
2251 TTCTTATACA TACTGAAGGA GGGAAAGGCG AAGGGAAGCT TCGGCATAGA
2301 CGTCGCAAAA CTCGCGGGAC TGCCCCGAGG AGTTGTAAGG GAAGCAAAA
2351 AGATACTGAA GGAGCTTGAA GGGGAAAAAG GAAAGCAGGA AGTTCTCCCC
2401 TTCCTTGAGG AGACCTATAA AAAGTCCGTT GATGAAGAGA AGCTGAACCT
2451 TTACGAAGAG ATAATAAAGG AGATAGAGGA GATAGATATA GGAACACGA
2501 CTCCTGTAA AGCCCTGCTC ATCCTTGCGG AGTTAAAGGA AAGGATAAAG
2551 AGCTTTATAA AGAGGTGA

```

G + C CONTENT: 47%

FIGURE 1

Apy MutS PROTEIN SEQUENCE

1 MGKEEKELTP MLAQYHQFKS MYPDCLLLFR LGDFYELFYE DAVVGSKELG
51 LVLTSRPAGK GRERIPMCGV PYHSANNYIA KLVNKGYSVA ICEQVEDPSK
101 AKGIVKRDVI RVITPGTFFE RETGGLCSLY RKGKSYLVSY LNLSVGFIG
151 AKVKEEELID FLSKFNIREV LVKKGEKLPE KLEKVLKLHI TELEEEFFEE
201 GKEELLKDYG VPSIKAFGFQ DEDLSLSLGA VYRYAKATQK SFTPLIPKPK
251 PYVDEGYVKL DLKAVKGLEI TESIEGRKDL SLFKVVDRTL TGMGRRRLRF
301 RLLNPFRSIE RIRKVQEAWE ELINKREVLN EIRKTLEGMS DLERLVSRIE
351 SNMASPRELI HLKNSLRKAE ELRKILSLLD SEIFKEIEGS LLNLNKVADL
401 IDKTLVDDPP LHVKEGGLIK PGVNAYLDEL RFIRENAEKL LKEYEKKLKK
451 ETGIQSLKIG YNKVMGYIIE VTKANVKYVP EHFRRRQTLS NAERYTTEEL
501 QRLEEKILSA QTRINELEYE LYRELREEVV KELDKVGNNA TLIGEVDYIQ
551 SLAWLALEKG WVKPEVHEGY ELIIEEGKHP VIEEFTKNYV PNDTKLTEEE
601 FIHVITGPNM AGKSSYIRQV GVLTLAHTG SFLPVKSARI PLVDAIFTRI
651 GSGDVLALGV STFMNEMLDV SNILNNATKR SLIILDEVGR GTSTYDGAII
701 SKAIVKYISE KIGAKTLLAT HYLELTELER KVKGVKNYHM EVEETDEGIR
751 FLYILKEGRA KGSFGIDVAK LAGLPEEVVR EAKKILKELE GEKGKQEVLP
801 FLEETYKKSIV DEEKLNFYEE IIEIEEIDI GNTTPVKALL ILAELKERIK
851 SFIKR*

$$M_r = 97655$$

FIGURE 2

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Tma MutS CODING SEQUENCE

```

1  GTGAAGGTAA CTCCCCTCAT GGAACAGTAC CTGAGAATAA AAGAACAGTA
51  CAAAGATTCC ATTCTGCTGT TTCGACTGGG AGATTTTAC GAGGCGTTTT
101 TCGAAGACGC AAAGATCGTT TCGAAGGTTT TGAACATAGT TCTCACAAGA
151 AGGCAGGACG CTCCCATGGC GGGCATCCCG TACCACGCGC TGAACACCTA
201 CCTGAAAAAG CTCGTGGAAG CGGGCTACAA GGTGGCAATC TGCGATCAAA
251 TGGAGAACC TTCGAAGTCG AAGAAATTGA TCAGAAGGGA AGTCACGCGC
301 GTTGTCACTC CCGGCTCCAT CGTAGAGGAT GAGTTTCTCA GCGAAACGAA
351 CAACTACATG GCCGTTGTCT CAGAAGAGAA AGGACGGTAC TGTACGGTTT
401 TCTGTGATGT CTCGACAGGT GAGGTCTCTG TTCATGAAAG TTCAGACGAA
451 CAGGAAACTT TGGACCTGCT GAAGAATTAC TCCATTTCCC AGATCATCTG
501 TCCAGAGCAC CTGAAATCTT CTTTGAAGGA ACGCTTTCCA GGTGTTTACA
551 CAGAAACCAT AAGCGAGTGG TATTTCTCAG ATCTGGAAGA AGTGGA AAAA
601 GCCTACAATC TGAAAGACAT TCATCATTTT GAGCTTTCGC CCCTTGCGCT
651 GAAAGCCCTT GCGGCGCTGA TAAAGTATGT CAAGTACACG ATGATCGGGG
701 AAGATCTGAA TCTGAAACCC CCTCTTCTCA TCTCCCAGAG AGACTACATG
751 ATACTCGATT CCGCAACGGT GGAAAATCTT TCTTGGATTC CCGGTGACAG
801 GGGAAAGAAT CTTTTCGATG TGCTGAACAA CACGGAAACT CCTATGGGGG
851 CTCGTCTTGG GAAAAAGTGG ATTCTCCACC CTCTGGTCGA CAGAAAACAG
901 ATCGAAGAAA GGCTCAAGGC TGTGGAAGA CTGGTGAACG ACAGGGTGAG
951 CCTGGAGGAG ATGAGGAACC TTCTTTCGAA CGTGAGGGAT GTGGAGCGGA
1001 TCGTTTCGCG GGTGGAGTAC AACAGTCCG TTCCAGGGA TTAGTGCCA
1051 CTCAGAGAGA CACTGGAGAT CATCCCGAAA CTGAACGAAG TTCTTCAAC
1101 CTTCGGTGTG TTCAAGAAAC TCGCTTTCCC GGAAGGACTG GTTGATCTGC
1151 TTCGAAAAGC CATTGAAGAT GATCCGGTGG GAAGCCCCGG CGAGGGAAAA
1201 GTTATAAAGA GAGGATTCTC ATCTGAACTC GACGAATACA GGGATCTTCT
1251 GGAACATGCC GAAGAGAGGC TCAAAGAGTT CGAGGAGAAG GAGAGAGAAA
1301 GAACAGGCAT CCAAAAACCTG CGGGTGGAT ACAACCAGGT TTTTGGTTAC
1351 TACATAGAGG TGACGAAGGC GAATCTGGAT AAGATTCCC GACGATTACGA
1401 AAGAAAACAA AACTCGTCA ATTCTGAAAG ATTCATACA CCCGAATTGA
1451 AGGAGTTCGA GACAAAGATA ATGGCCGCTA AAGAGAGAAT AGAAGAACTG
1501 GAAAAGGAAC TCTTCACAAG CGTGTGCGAA GAGGTGAAAA AGCACAAAGA
1551 AGTTCTCCTT GAGATCTCGG AGGATCTGGC AAAGATAGAT GCGCTTTCGA
1601 CGTTAGCATA CGACGCTATT ATGTACAACT ACACAAAACC CGTCTTTTCA
1651 GAAGACAGAC TGGAGATCAA AGGTGGAAGA CACCCGGTCG TTGAAAGGTT
1701 CACACAGAAT TTTGTTGAAA ACGATATTTA CATGGACAAC GAGAAGAGAT
1751 TTGTGGTAAT AACGGGTCCC AACATGAGCG GGAAGTCCAC TTTCATCAGA
1801 CAGGTGGGTC TCATATCCCT CATGGCGCAG ATAGGATCGT TTGTGCCGGC
1851 GCAGAAGGCG ATTCTTCCAG TGTTGACAG GATTTTCACG CGAATGGGTG
1901 CCAGAGACGA TCTCGCTGGT GGTAGAAGTA CGTTCCTTGT CGAGATGAAC
1951 GAGATGGCGC TCATCCTTCT GAAATCAACA AATAAGAGTC TGGTTCTCCT
2001 GGACGAGGTG GGAAGAGGTA CAAGCACCCA GGACGGCGTC AGCATAGCCT
2051 GGGCAATCTC AGAGGAACTC ATAAAGAGAG GATGTAAGGT GCTGTTTGCC
2101 ACTCATTTCA CGGAACTCAC GGAACCTGAA AAACACTTTC CGCAGGTTCA
2151 GAACAAAACC ATTCTGGTAA AAGAAGAAGG CAAAACGTG ATATTACCC
2201 ACAAGGTGGT GGACGGTGTG GCAGACAGAA GTTACGGAAT AGAGGTCGCA
2251 AAGATAGCGG GTATTCTCTG CAGGGTTATA AACAGAGCCT ATGAAATTCT
2301 GGAGAGGAAT TTCAAAAACA ACACGAAGAA AAACGAAAAA TCGAACAGAT
2351 TCAGTCAGCA AATTCCTCTC TTTCTGTTT GA

```

G + C CONTENT: 47%

FIGURE 3

Tma MutS PROTEIN SEQUENCE

1 VKVTPLMEQY LRIKEQYKDS ILLFRLGDFY EAFFEDAKIV SKVLNIVLTR
51 RQDAPMAGIP YHALNTYLKK LVEAGYKVAI CDQMEEPSKS KKLIRREVTR
101 VVTPGSIVED EFLSETNNYM AVVSEEKGRY CTVFCDVSTG EVLVHESSDE
151 QETLDLLKNY SISQIICPEH LKSSLKERFP GVYTETISEW YFSDLEEVEK
201 AYNLKDIHHF ELSPLALKAL AALIKYVKYT MIGEDLNLKP PLLISQRDYM
251 ILDSATVENL SWIPGDRGKN LFDVLNNTET PMGARLGKKW ILHPLVDRKQ
301 IEERLKAVER LVNDRVSLEE MRNLLSNVRD VERIVSRVEY NRSVPRDLVA
351 LRETLEIIPK LNEVLSTFGV FKKLAPPEGL VDLLRKAIED DPVGSPGEGK
401 VIKRGFSSEL DEYRDLLEHA EERLKEFEK ERERTGIQKL RVGYNQVFGY
451 YIEVTKANLD KIPDDYERKQ TLVNSERFIT PELKEFETKI MAAKERIEEL
501 EKELFTSVCE EVKKHKEVLL EISED LAKID ALSTLAYDAI MYNYTKPVFS
551 EDRLEIKGGR HPVVERFTQN FVENDIYMDN EKRFVVITGP NMSGKSTFIR
601 QVGLISLMAQ IGSFVPAQKA ILPVFDRIPT RMGARDLAG GRSTFLVEMN
651 EMALILLKST NKSLVLLDEV GRGTSTQDGV SIAWAISEEL IKRGCKVLFA
701 THFTELTELE KHFPQVQNKT ILVKEEGKNV IFTHKVVDGV ADRSYGIEVA
751 KIAGIPDRVI NRAYEILERN FKNNTKNGK SNRFSQQIPL FPV*

$$M_r = 91009$$

FIGURE 4

Tth MutS Sequence

```
1  AAGTCCACCT TCCTCCGCCG GACCGCCCTC ATCGCCCTCC TCGCCCAGAT
51 CGGGAGCTTC GCGCCCGCCG AGGGGCTGCT GCTTCCCCTC TTTGACGGGA
101 TC
```

FIGURE 5

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Taq MutS Sequence

1 AAGTCCACCT TTCTGCGCCA GACGGCCCTC ATCGCCCTCC TGGCCCAGGT
51 GGGGAGCTTC GTGCCC GCCG AGGAGGCCCA TCTTCCCCTC TTTGACGGCA
101 TC

FIGURE 6

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613
Apy KSSYIRQVG VLTLLAHTGS FLPVKSARIP LVDAI
Taq KSTFLRQTA LIALLAQVGS FVPAEEAHLP LFDGI
Tth KSTFLRRTA LIALLAQIGS FAPAEGLLLP LFDGI
Tma KSTFIRQVG LISLMAQIGS FVPAQKAILP VFDRI
595

FIGURE 7

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Apy MutL Coding sequence: Upper case

```

-60  gaattcttaa ggttctcaag ggctgttctt ttctcttttt ccttcctaatt ttaataacctc
   1  ATGTTTGTCA AAATCCTGCC CCCAGAGGTA AGGAGAAAGA TTGCAGCGGG AGAGGTTATA
  61  GACGCTCCCG TTGACGTTGT AAAAGAGCTT ATAGAGAACT CCCTTGACGC TAAGGCAACG
 121  AGGATTGAGA TTGAGGTCGT AAAAGGGGGG AAAAGACTTA TCAGAGTTAA GGATAACGGG
 181  ATAGGCATTC ATCCCGAGGA TATAGAAAAG GTCGTTTTAT CGGGAGCTAC GAGCAAGATA
 241  GAGAAGGAAA CGGACCTCCT CAATGTGGAA ACCTACGGAT TCAGGGGGGA AGCCCTGTAT
 301  TCCATCTCAA GCGTAAGCAA GTTCAGGCTA AGGTCAAGGT TTTACCAGGA AAAGGAAGGA
 361  AGGGAGATAG AAGTTGAGGG GGGAAACGCTA AAAAGCGTCA GAAGAGTAGG AATGGAAGTT
 421  GGGACGGAAG TTGAGGTTTA CGACCTCTTT TTTAACCTCC CCGCAAGGAA GAAATTTTTA
 481  AGAAAGGAAG ACACCGAAAG GAGAAAGATA ACGGAGCTCG TAAAGGAGTA TGCCATAACA
 541  AACCCCCAGG TTGACTTTCA CCTCTTTTCC GAAGGAAAGG AAACCTTAA CCTGAAGAAG
 601  AAGGACCTAA AAGGGAGAAT TGAGGAAATC TTTGAGTCAA TTTTGAAGA AGAAAGCTCG
 661  GAAAGGGAAG GAATAAAGGT AAGAGCCTTC ATATCAAGAA ACCAGAAAAG GGGAAAGTAT
 721  TACCTCTTCG TAAACTCAAG ACCAGTTTAC AACAAAACT TAAAAGAATA CCTAAAGAAA
 781  ACCTTCGGTT ATAAAACGAT AGTCGTGCTG TTCATTGATA TTCCCCCCTT TCTCGTTGAC
 841  TTTAACGTTC ACCCCAAAAA GAAAGAGGTA AAGTTTTTAA AAGAGCGAAA GATTTACGAA
 901  CTCATAAGGG AACTCTCTTC CAGAAAACAC ACAATCCTTG AGATACCTAC ACTTAATCAG
 961  AAAACCGAAA GTTATAAACC GACATACGAG GTTATAGGTC AACTAAACGA AACCTTTATT
1021  CTCGTAAGCG ACGGGAACCT TTTATACTTC ATAGACCAGC ACCTTCTTGA TGAGAGAATA
1061  AACTACGAGA AAAATGGAAA CGAAGAACTT GCCTGCAGAA TTTCCGTAAA AGCGGGGGAA
1121  AAATTAACAA ACGAAAAGAT AAAAGAACTC ATAAAGGAAT GGAAAAAGCT TGAAAACCCC
1201  CACGTATGTC CCCACGGCAG ACCTATATAC TACAAACTCC CCTTAAAGGA AGTATACGAA
1261  AAGCTCGGAA GGAGTTTTTA Aggtaaaatt ctatagaccc aatgttcagc attaagttct

```

Figure 8

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Tma MutL Coding sequence: Upper case

```

-60          ttttttctgg atgttaaaat tttcagggag atcgagtgga gaggtgttct
  1  GTTTTGAGAA TAAAAAGACT TCCCAGAGAGC CTCGTCAGAA AAATCGCCGC GGGTGAGGTG
 61  ATTCACAATC CATCTTTCGT TCTGAAAGAG CTTGTAGAAA ACAGTCTGGA CGCGCAGGCC
121  GACAGGATAG TTGTTGAGAT AGAAAACGGT GGAAAGAACA TGGTAAGAGT ATCCGACAAT
181  GGAATCGGGA TGACCAGAGA AGAGGCACCT CTGGCAATAG AACCTTACAC GACGAGCAAG
241  ATAGAGAGCG AGGAAGATCT GCACAGGATC AGAACTTACG GTTTCAGAGG TGAAGCGCTT
301  GCTTCGATTG TGCAGGTCAG CAGAGCCAAG ATCGTGACAA AAACGGAAAA AGACGCACTC
361  GCAACACAGT TGATGATTGC TGGGGGGAAA GTGGAAGAAA TCTCGGAAAC CCACAGGGAT
421  ACCGGCACCA CCGTTGAGGT GAGAGATCTC TTCTTCAACC TACCCGTCCG GAGAAAATCT
481  CTGAAGTCCT CTGCCATCGA GTTGAGAATG TGTCGTGAGA TGTTTGAAAG ATTCTCTCTT
541  GTACGAAACG ACGTTGATTT TGTATTACC TCAGATGGAA AGATAGTCCA TTCCTTTCCA
601  AGAACACAGA ACATCTTTGA AAGAGCTCTC CTGATCCTTG AAGATCTGAG AAAAGGTTAC
661  ATCACGTTTCG AAGAGGAATT ATCCGGCCTG AGGATAAAGG GAATAGTTTC ATCCCGCGAG
721  GTGACAAGAT CCAGCAGAAC GGGAGAGTAT TTCTACGTGA ACGGTCGTTT TGTGGTTTCC
781  GAAGAACTCC ACGAAGTACT CATGAAAGTT TACGATCTTC CAAAGAGAAG CTATCCCGTC
841  GCGGTTCTTT TCATAGAGGT AAATCCGGAA GAACTCGACG TGAACATACA CCCTTCGAAA
901  ATCGTGGTGA AATTTCTCAA CGAAGAAAAG GTGAAAAGA GTTTGGAAGA AACCCTCAAA
961  AGAAATCTGG CACGGAAATG GTACAGGTCG GTTGCGTACG AAGAAATATC CTCCCGTGCG
1021 CTGAGCGTGG CAGAAGCACC ATCCCACAGA TGGTTTTTGG TCAAGGGTAA GTACGCTGTC
1081 GTTGAAGTGG AAGATGGTTT GCTCTTTGTG GATCTTCATG CTCTCCACGA ACGAACGATT
1141 TACGAAGAAA TCCTTTCGAA AAAAGCTGG GGGAAAAGAC GGGTGAAAAG GAACATAACA
1201 GTTGTGCTAT CAAGGGAAGA AAAACAAAA CTGGAAGAAT ACGGATTCTC CTTTCAAGGA
1261 GAAGAAGGAG CTTTGAAAGT CATTGAAATC CCTGAGTTCC TCACCGAAGA CGTTGTGGAG
1321 GAATTTTTCA GGGACTTCCC AGTTGATGAA AAAGTGAAGG AAAGAATAGC CTTTGCCGCT
1381 TGTAAACTTG CCACTAAATC CGGAGAATC GACGAAGAGA TCGCATCGAA ACTGCTGGAT
1441 GTCTTTTTCA AGAAGCGGTT TGAAAGATGT CCTCACGGAA GGCCGATTTC TTTCAAGATC
1501 AGCTATGAGG ACATGGACCG ATTTTTCGAG CGTTAAccca ttttcaccac gttgacgtca
1561 gcggtgaaaa ccaggccatc gaagtctatg

```

Figure 9

Apy. Pe IDIGNTPVKALLILAELEKRIKSFIKR*

Figure 10

A. pyrophilus and *T. maritima* versus *S. pneumoniae* HexB and *E. coli* MutL (PILEUP)

1	Apy	.MEVKILPPE	VRKIAAGEV	IDAPDVVKE	LIENSIDAKA	TRIEIEVVKG	50
	Tma	MLRIKRLPES	LVRKIAAGEV	IHNPSVLKE	LVERSLDAQA	DRIVVEIENG	
	Spn	MSHIIIEPEM	LANQIAAGEV	IERPASVCKE	LVENAIDAGS	SQIIIEIEEA	
	Eco	.MPIQVLPPQ	LANQIAAGEV	VERPASVCKE	LVENSIDAGA	TRIDIDIERG	
51	Apy	GKRLIRVKDN	GIGIHPEIDIE	KWLSCGATSK	IEKETDILLNV	ETYGERGEAL	100
	Tma	GKNMVRVSDN	GIGMTREREAL	LAIEPYTTSK	IESEEDLHRI	RTYGERGEAL	
	Spn	GLKKVOITDN	GHGIAHDEVE	LALRRHATSK	IKNOADLFRI	RTLGRERGEAL	
	Eco	GAKLIRIRON	CGGIKKDELA	LALARHATSK	IASLDDLEAI	ISLGRERGEAL	
101	Apy	YSISSVSKFR	LRSRYQOEKE	GREIEVEGGT	LK SVRRVGH	EVGTETEVEVD	150
	Tma	ASIVQVSRAK	IVTRTEKDAL	ATQMIAGGK	VE.EISETHR	DTGTTVEVRD	
	Spn	PSIASVSVLT	LLTAVDGGASH	GTKLVARGGE	VE.EVIPATS	PVGTKVCVED	
	Eco	ASISSVSRLT	LTSRTAEQQE	AWQAYAEGRD	MNVTVKPAAH	PVGTTLEVLID	
151	Apy	LFENLPARKK	FLRKEDTERR	KITELVKEYA	ITNPQVDFHL	FSEGETLNL	200
	Tma	LFENLPVRRK	SLKSSAIELR	MCREMFERFV	LVRNDVDVRF	TSDGKIVHSF	
	Spn	LFENTPARLK	YMKSQQAELS	HIIDIVNRLG	LAHPEISFSL	ISDGKEMTRT	
	Eco	LFYNTPARRK	FLRTEKTEFN	HIDEIIRRIA	LARFDTVTLN	SHNGKIVRQY	
201	Apy	...KKKOLKG	RIEIEFESI.FEES	SEREGIKVRA	FISRNQ....	250
	Tma	..PRTONIFE	RALLILEDLR	KGYITFEEL	S....GLRIKG	IVSSREVTRS	
	Spn	..ACTGOLRQ	AIAGIY.GLV	SAKMMIEIEN	SD.LDFEISG	FVSLPELTRA	
	Eco	RAVPEGGOKE	RRLGAIICGTA	FLEQALAE.	WQHGDLTIRG	WVADPNHTTP	
251	Apy	KRGKY.YLHV	NSRPVYNKNI	KEYLKKTTFG.	.YK....TIV	VLFIDIPPEL	300
	Tma	SRTGE.YFYV	NGRFWSEEL	HEVLWKVYD.	.IPKRSYPA	VLFIEVNPPE	
	Spn	NRNYI.SLFI	NGRYIKNFLL	NRAILDGFGS	KLMVGRFPLA	VIHIIIDPYL	
	Eco	ALAEIQCYV	NGRMRRDLRI	NHAIROACED	KLGADQOPAF	VLYLEIDPHQ	
301	Apy	VDNVNHPKKK	EVKELKERKIYELIR	ELSSRKHTIL	EIPTLNKTE	350
	Tma	LDVNIHPSKI	VVKFLNEEKV	KKSLEETLKR	NLARKHYRSV	AYEEISSRAL	
	Spn	ADVNVHPTKQ	EVRISKEKEL	MTLVSEAIA	SLKEQTLIPD	ALENLAKSTV	
	Eco	VDNVNHPAKH	EVRFHQSRV	HDFIYQGVLS	VLQOQLETP	PLDDEPQAP	
351	Apy	SY.K.....	400
	Tma	SVAE.....	
	Spn	RNRKVEQTI	LPLKENTLYY	EKTEPSRPSQ	TEVADYQVEL	TDEGQDLTLF	
	Eco	RSIFENRVAA	GRNHFAEPAA	REPVPRTYP	APASGSRPAA	P.....	
401	Apy	450
	Tma	
	Spn	AKETLDRLTK	PAKLHFAERK	PANYDQOLDHP	ELDLASIDKA	YDKLREERAS	
	EcoWP	NAQPGYQKQQ	GEVYROLLQT	PAPMOKLKAP	EPOEPALAA	
451	Apy	..PTYEVIGQ	LNETFILVSD	GNFLYFIDQH	LLDERINY..	500
	Tma	..APSHRNFL	VNGKYAVVEV	EDGLFVDLH	ALHERTIYEE	ILSKKSWGKR	
	Spn	SPELEFFGQ	MHGTYLFAQG	RDGLYIIDOH	AAQERVYEE	YRESIGNVDDQ	
	Eco	SQSFGRVLT	VHSDCALLER	DGNISLUSLP	VAERWLQRAQ	LTPGEAPV..	
501	ApyEKNGNEE	LACRISV.KA	G.....E	550
	Tma	RVKRNITVVL	S.....	..REEKQLEE	YGFSFOG.EE	GALKVIEIPE	
	Spn	SQOQLVPYI	FEPPADDALR	LKERHPLLEE	VGVELAEYGE	NOFILREHPI	
	Eco	CAOPLLIPLR	LKVSABEESA	LEKAQSALAE	LGIDFQS.DA	QHVITRAVPL	
551	Apy	KLTNEKIKE.L	IKEM...KKL	ENP.....	600
	Tma	FLTEDVVEE.F	FRDPVDEKL	KERIALAACK	LATKSGEFDE	
	Spn	WMAEEIEESG	IYEMCDMLLL	TKEVSIRKYR	AEALAIMMSCK	RSIKANHRID	
	Eco	PLRQONLQIL	IPELIG..YL	AKQSVFEP..	GNTAQMIARN	LMSEHAQWSM	
601	ApyHV..C	PHG...RPIY	YKLPPLKEVYE	KLGRSF..	650
	Tma	EIASKLLDVF	FKKRFER..C	PHG...RPIS	FKIS.....YE	DHRRFFER*	
	Spn	DHSARQLLYQ	LSQCDNPYNC	PHG...RPVL	VHFTKSDM.E	KMFERRIOENH	
	Eco	AQAITLLADV	ERLCPQLVKT	PPGGLLQSV	LHPAIKALKD	E.....	
651	Apy	
	Tma	
	Spn	TSLRELKGY*	
	Eco	

Figure 11

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```

1  GAATTCGATC ACCTGCAAGA AGTCATCAAG CGCCTGGCCC TGGCCCGTTT
51  CGACGTGGCC TTTCACCTGC GCCACAATGG CAAGACCATC CTCAGCCTGC
101 ACGAAGCCAA CGACGACGCC GCCCGTGCTC GGCGGGTGGC GGCGGTGTGT
151 GGCAGCGGGT TCCTGGAGCA GGCCTGCCC ATTGAGATCG AGCGCAATGG
201 CTTGAGGTTG TGGGGCTGGG TCGGGTTGCC GACGTTCTCC CGCAGCCAGG
251 CCGATTGCA GTATTTCTTT GTGAACGGCC GGGCGGTCCG CGACAAACTG
301 GTGGCCCATG CGGTGCGCCA GGCTTATCGC GATGTGCTGT TCAACGGGCG
351 ACACCCGACT TTTGTGCTGT TCTTTGAGGT TGACCCTTCG GTGGTC

```

Figure 13

```

151                                     200
E. coli  LFYNTPARRK FLRTEKTEFN HIDEIIRRIA LARFDVTINL SHNGKIVRQY
T. ther  ..... EFD HLQEVIKRLA LARFDVAFHL RHNGKTILSL
S. pneu  LFFNTPARLK YMKSQQAELS HIIDIVNRLG LAHPEISFSL ISDGK...EM

201                                     250
E. coli  RAVPEGGQKE RRLGAICGTA FLEQALAIEW QHGDLTLRGW VADPNHTTPA
T. ther  HEANDDAARA RRVAAVCGSG FLEQALPIEI ERNGLRLGW VGLPTF.SRS
S. pneu  TRTAGTGQLR QAIAGIYGLV SAKKMIEIEN SLDLFEISGF VSLPEL.TRA

251                                     300
E. coli  LAEIQYCYVN GRMMRDRLIN HAIRQACEDK LGADQQPAFV LYLEIDPHQV
T. ther  QADLQYFFVN GRAVRDKLVA HAVRQAYRDV LFNGRHPTFV LFFEVDPSVV
S. pneu  NRNYISLFIN GRYIKNFLN RAILDGFGSK LMVGRFPLAV IHIHIDPYLA

```

Figure 14

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/11567

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C12N15/10 C12N1/21 C07K14/195 C12Q1/68
C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K C12Q C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J.A. MANKOVICH ET AL.: "Nucleotide sequence of the Salmonella typhimurium mutL gene required for mismatch repair: Homology of MutL to HexB of Streptococcus pneumoniae and to PMS1 of the yeast Saccharomyces cerevisiae" J. BACTERIOL., vol. 171, no. 10, October 1989, AM. SOC. MICROBIOL., BALTIMORE, US;, pages 5325-5331, XP002042222 see the whole document --- -/--	1-9



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

30 September 1997

Date of mailing of the international search report

15.10.97

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Hornig, H

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/11567

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M. PRUDHOMME ET AL.: "Nucleotide sequence of the Streptococcus pneumoniae HexB mismatch repair gene: Homology of hexB to MutL of Salmonella typhimurium and to PMS1 of Saccharomyces cerevisiae" J. BACTERIOL., vol. 171, no. 10, October 1989, AM. SOC. MICROBIOL., BALTIMORE, US;; pages 5332-5338, XP002042223 see the whole document ---	1-9
A	W. KRAMER ET AL.: "Cloning and nucleotide sequence of DNA mismatch repair gene PMS1 from Saccharomyces cerevisiae: Homology of PMS1 to procaryotic MutL and HexB" J. BACTERIOL., vol. 171, no. 10, October 1989, AM. SOC. MICROBIOL., BALTIMORE, US;; pages 5339-5346, XP002042224 see the whole document ---	1-9
A	H.-C. TIFFANY ET AL.: "Nonconserved segment of the MutL protein from Escherichia coli K-12 and Salmonella typhimurium" NUCLEIC ACIDS RESEARCH, vol. 20, no. 9, 11 May 1992, IRL PRESS LIMITED, OXFORD, ENGLAND, page 2379 XP002042225 see the whole document ---	1-9
A	M. GRILLEY ET AL.: "Isolation and characterization of the Escherichia coli mutL" J. BIOL. CHEM., vol. 264, no. 2, 15 January 1989, AM. SOC. BIOCHEM. MOL. BIOL., INC., BALTIMORE, US, pages 1000-1004, XP002042226 see the whole document ---	1-9
A	L. WORTH ET AL.: "Mismatch repair proteins MutS and MutL inhibit RecA-catalyzed strand transfer between diverged DNAs" PROC. NATL. ACAD. SCI., vol. 91, April 1994, NATL. ACAD. SCI., WASHINGTON, DC, US;; pages 3238-3241, XP002042227 cited in the application see the whole document ---	1-20
1 5 A	WO 95 16793 A (UNIV OREGON HEALTH SCIENCES ;DANA FARBER CANCER INST INC (US); BAK) 22 June 1995 see the whole document ---	1-9
	--- -/--	

INTERNATIONAL SEARCH REPORT

Int. Appl. No.

PCT/US 97/11567

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TAKAMATSU S ET AL: "MISMATCH DNA RECOGNITION PROTEIN FROM AN EXTREMELY THERMOPHILIC BACTERIUM, THERMUS THERMOPHILUS HB8" NUCLEIC ACIDS RESEARCH, vol. 24, no. 4, 15 February 1996, pages 640-647, XP002014198 cited in the application see the whole document ---	1-9
A	I. BISWAS AND P. HSIEH: "Identification and characterization of a thermostable MutS homolog from Thermus aquaticus" J. BIOL. CHEM., vol. 271, no. 9, 1 March 1996, AM. SOC. BIOCHEM. MOL. BIOL., INC., BALTIMORE, US, pages 5040-5048, XP002011497 cited in the application see the whole document ---	1-9
A	WO 95 12688 A (US BIOCHEMICAL CORP) 11 May 1995 see the whole document ---	1-20
P,X	J.G. WETMUR ET AL.: "Aquifex pyrophilus DNA mismatch repair protein (MutL) gene, complete cds." EMBL SEQUENCE DATABASE, 4 October 1996, HEIDELBERG, BRD, XP002042228 Accession no. U71052 ---	1-9
P,X	J.G. WETMUR ET AL.: "Thermotoga maritima DNA mismatch repair protein (MutL) gene, complete cds." EMBL SEQUENCE DATABASE, 4 October 1996, HEIDELBERG, BRD, XP002042229 Accession no. U71053 ---	1-9
P,X	A. YAMAMOTO ET AL.: "Thermus aquaticus HexB/MutL homolog (mutL) gene, complete cds." EMBL SEQUENCE DATABASE, 20 January 1997, HEIDELBERG, BRD, XP002042230 Accession no. U50453 ---	1-3,6,8, 9
P,A	WO 96 39525 A (MOUNT SINAI MEDICAL CENTER OF) 12 December 1996 cited in the application see the whole document -----	1-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/11567

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9516793 A	22-06-95	AU 1442495 A EP 0760867 A	03-07-95 12-03-97
WO 9512688 A	11-05-95	US 5556750 A US 5459039 A CA 2175490 A EP 0788555 A JP 9504437 T	17-09-96 17-10-95 11-05-95 13-08-97 06-05-97
WO 9639525 A	12-12-96	NONE	